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PRINCIPAL INVESTIGATOR: Anna Lokshin, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213

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14. ABSTRACT

We have identified two panels of SEREX antigens associated with breast cancer in pre- and post-menopausal women. Suspended bead arrays were generated using Luminex microspheres. Serum samples from patients with breast cancer, patients with benign breast lesions and healthy volunteers were collected and concentrations of serum breast cancer biomarkers samples were determined. Novel ADEPT bioinformatics algorithm was utilized to analyze the results. In the postmenopausal group, a 23-biomarker panel was selected using the projection pursuit technique correctly classifying 84% of the test set observations, with a sensitivity of 85% at a specificity of 83%. In the premenopausal group, a 20-biomarker panel was identified providing sensitivity of 88% at a specificity of 89% increasing that of mammography for this population. Parallel analysis of these samples using SELDI-TOF MS technology in combinations with BI-RADS scores allowed for correct classification of 80% of patients. The presented data demonstrate that simultaneous testing of a panel of breast cancer-associated serum antigens identified by SEREX using suspended bead array technology allows establishing comprehensive antigenic profiles with high predictive power for breast cancer.

15. SUBJECT TERMS
Breast Cancer

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INTRODUCTION

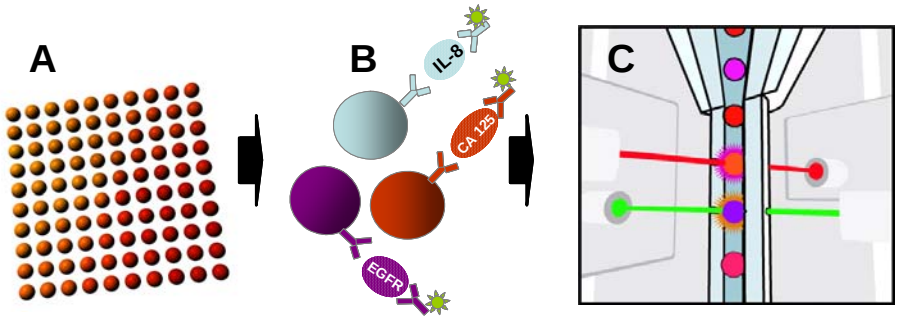
Breast cancer is one of the most common causes of cancer-related deaths in women. It is estimated that in the United States over 40,410 will die from breast cancer this year. Breast cancer outcomes cannot be improved without enhanced screening and prevention efforts. The occurrence of breast cancer is usually suggested either by physical exam or as a result of screening mammography. However, mammography misses up to 40% of cancers in premenopausal women who have dense breast tissue. In addition, due to false positive mammograms, 70% to 80% of breast biopsies from patients with an abnormal radiographic image demonstrate a benign process. *Therefore, additional detection methods for premenopausal women are needed to 1) identify breast tumors at early, ideally pre-invasive, stages; 2) detect breast cancer at close to 100% sensitivity; and 3) provide a substantially higher degree of specificity than presently afforded by screening mammography.* A clinically useful blood test for early detection of breast cancer would be very helpful, but the ideal serological marker for malignancy of the breast has not yet been identified. In addition, such test could be potentially applied to early detection of recurrence in patients treated for breast cancer. The proposed study was based on the *hypothesis* that a combination of several serum biomarkers would provide superior diagnostic power. To test this hypothesis, we utilized a novel multiplexing xMAP® technology (Luminex Corp., Austin, TX) in combination with SELDI-TOF MS proteomic platform for screening of a broad range of serum biomarkers in breast cancer patients.

BODY

Task 1. Generation of protein microarrays of breast cancer-associated antigens as identified by SEREX.

Luminex xMAP® multiplexed platform. Recently, LUMINEX Corporation introduced a novel protein array system (xMAP® for Multianalyte Profiling), which allows for simultaneous quantitation of up to 100 soluble analytes in one sample. xMAP® technology uses polystyrene microspheres internally dyed with differing ratios of two spectrally distinct fluorophores to create a family of 100 differentially spectrally addressed bead sets (Figure 1A). Each of the 100 spectrally addressed bead sets can be conjugated with a capture antibody specific for a unique target analyte (Figure 1B). In a multiplexed assay, antibody-conjugated beads are allowed to react with sample (plasma, serum or other biofluid specimen). After washing, secondary, or detection, antibodies are added to a microtiter plate well to form a capture sandwich immunoassay (Figure 1B). The bead suspension is then analyzed by the fluorometric array reader, which obtains two fluorescence readings for every single bead: one that identifies a bead as a member of one of the 100 possible sets, and another that measures the amount of fluorescent dye, typically phycoerythrin (PE), bound to the detection antibody in the assay (Figure 1C). The amount of PE fluorescence is proportional to the amount of analyte captured in the immunoassay. Bio-Plex Manager software correlates each bead set to the assay reagent that has been coupled to it. Extrapolating to a standard curve allows quantitation of each analyte in the sample. Using the xMAP® assay, thousands of beads can be analyzed in seconds, allowing up to 100 analytes to be measured in a 96-well microplate in one hour. In addition, since the fluorescence from each bead is measured independently, sufficient statistics are accumulated to allow for assaying each sample in one well and not in duplicates. *Based on the above features of the xMAP® system, we considered it to be highly suitable for multimarker screening in breast cancer and have utilized this technology in this project.*

Figure 1.
Luminex xMAP® technology



Generation of multiplexed bead-based protein microarrays of breast cancer-associated antigens as identified by SEREX.. We have developed multiplexed assays for 30 cancer antigens identified by SEREX, CA 15-3, CA 19-9, CA 125, CEA, CA 72-4, AFP, ErbB2, EGFR, kallikreins 6,8,10, Fas, FasL, Cyfra 21-1, TPA/TPS, IGFBP1, S100, angiostatin, SSC, ULBP1,2,3, βHCG, MICA, HE4, SMRP, mesothelin, SAA, and TTR. The procedure we used for developing these new assays in the UPCI Luminex Core Facility was as follows. Capture antibodies were monoclonal, and detection antibodies were polyclonal. Capture antibodies were biotinylated using EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The extent of biotin incorporation was determined using the HABA assay and was typically ~20 moles of biotin per mole of protein. Capture antibody was covalently coupled to carboxylated polystyrene microspheres number 74 purchased from Luminex Corporation (Austin, TX). Covalent coupling of the capture antibodies to the microspheres was performed following the procedures recommended by Luminex. Coupling efficiency of the monoclonal antibodies was tested by staining 2000 microspheres with PE-conjugated goat anti-mouse IgG (BD Biosciences, San Diego, CA). The assay was further optimized for concentration of the detection antibody and for incubation times. The results of analytical assay validation are presented in Table 3 for representative assays. Intra-assay variability, expressed as a coefficient of variation, was calculated based on the average for 10 patient samples and measured twice at two different time points. Every individual Luminex assay that was used in our study has been validated according to commercial standards for: a.) sensitivity, b). inter- and intra-assay reproducibility, c.) % recovery from serum, and d.) against conventional single analyte ELISA. Additionally, performance of each assay singly is compared to that when multiplexed to make sure that there is no cross-reactivity. The results of assay validation shown in Table 1 confirm that each individual assay demonstrated high sensitivity and reproducibility, as well as recovery and correlation with appropriate ELISA. Finally, these microspheres were combined into a multiplex panel and the performance of each marker in the multiplexed panel was re-validated versus the performance of the same marker in single-plex format.

Table 1. Validation of multiplexed xMAP® assays developed in UPCI Luminex Core Facility

Biomarkers	CA 19-9	CA 125	CEA	CA 15-3	ErbB2	EGFR	Fas	FasL	Cyfra 21
Sensitivity	20 IU/ml	5 U/ml	50 U/ml	1 IU/ml	50 pg/ml	5 pg/ml	15 pg/ml	50 pg/ml	300 pg/ml
Recovery (serum), %	96	101	99	98	100	98	98	95	89
Intra-assay variation, %CV	8.6	6.0	8.2	7.9	6.5	7.1	5.5	6.2	5.1
Inter-assay variation (%CV)	5.4	6.2	6.4	5.1	7.1	6.8	6.1	3.2	5.3
Correlation with ELISA	0.97	0.98	0.98	0.97	0.99	0.98	0.99	0.99	0.98

Task 2. Collect serum samples from patients with breast cancer, ductal carcinoma in situ, women at high risk for breast cancer, patients with benign breast lesions and healthy volunteers.

Sera were collected from 120 patients with early stage breast cancer, 100 women with benign breast masses, and 109 age-matched healthy controls (Table 2). Sera were also collected from patients with ductal carcinoma *in situ*. Sera from women at high risk for breast cancer are presently being collected.

Table 2. Patient Characteristics

Patient Group	Postmenopausal Age	Histology	Premenopausal Age	Histology

Control	N=109 Range 48-84 Median 60 Average 61.4		N=59 Range 22-50 Median 36.7 Average 37.2	
Breast Cancer	N=120 Range 49-82 Median 62 Average 63.2	Invasive ductal (n=28) Invasive lobular (n=5) Invasive metaplastic (n=1) Invasive tubular (n=2) Infiltrating ductal (n=14) Infiltrating lobular (n=3) Infiltrating mucinous (n=2) DCIS (n=23) Bilateral cancer (n=4) Invasive infiltrating ductal (n=49)	N=52 Range 30-49 Median 41.4 Average 41.5	Invasive ductal (n=16) Invasive lobular (n=2) Invasive tubular (n=1) Infiltrating ductal (n=12) Infiltrating lobular (n=1) Bilateral cancer (n=3) Invasive infiltrating Ductal (n=15) Inflamatory carcinoma (2)
Benign Tumors	N=100 Range 48-84 Median 60 Average 60.4	Fibrosis (n=3) Fibroadenoma (n=13) Fibrocystic changes (n=14) Benign breast tissue (n=5) Lipoma (n=3) Introductal papilloma (n=6) Introductal hyperplasia (n=4) Cyst (n=5) Others (n=46)	N=49 Range 21-52 Median 40.1 Average 38.3	Fibrosis (n=2) Fibroadenoma (n=8) Fibrocystic changes (n=10) Benign breast tissue (n=5) Lipoma (n=2) Intraductal papilloma (n=3) Cyst (n=2) Abscess (2) Others (n=15)

Task 3. Screen the antigen array and establish comprehensive antigenic profiles for breast cancer patients. Construct a clinical prediction rule to identify SEREX markers of breast cancer.

Screening the antigen array and establishing comprehensive antigenic profiles for breast cancer patients. Fifty-three biomarkers were identified by SEREX and included several groups of proteins with different biological functions related to tumor development, i.e. cancer antigens, CA 125, CA 15-3, CEA, AFP, CA 72-4; growth/angiogenic factors, VEGF, bFGF, IGFBPI, HGF, ErbB2, EGFR; apoptosis-related molecules, Fas, FasL, Cyfra 21-1; metastasis-related molecules, MMP-2, MMP-3, tPAI, sICAM, sVCAM, sE-selectin; cytokines, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, TNFR I, TNFR II, IFNγ, GM-CSF, G-CSF, M-CSF, IL-2R, IP-10, MCP-1, MIP-1α, MIP-1β, MIF, eotaxin, RANTES; hormones, βHCG; proteases: kallikreins 8 and 10; adipokines: resistin; as well as mesothelin, and myeloperoxidase (MPO).

Serum concentrations of AFP, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM, MPO, tPAI, MIF, MMP-2, resistin, CA 15-3, IL-8, ErbB2, sE-Selectin, sVCAM, and MMP-3 were significantly elevated in patients with breast cancer as compared to healthy controls ($p<0.05$ – $p<0.001$; Figure 2). Serum levels of other proteins were not statistically different in the three tested clinical groups. Next, the group with benign breast disease was compared to the breast cancer group. Serum concentrations of IL-6, IP-10, AFP, CA 15-3, CA 19-9, EGFR, MMP-2, and MIF were significantly lower and sFasL was significantly higher in women with benign breast disease as compared to patients with breast cancer ($p<0.05$ - $p<0.001$). Therefore, some biomarkers could potentially be utilized in a screening test, as they demonstrate significantly different expression patterns in the serum samples of patients from women with early stage breast cancer, women with benign masses, and age-matched healthy controls. However, none of the individual biomarkers was able to provide singly the classification accuracy higher than 40% sensitivity at 50% specificity.

Construction of a clinical prediction rule to identify SEREX markers of breast cancer. By developing of adaptive density estimator – Projection Pursuit (ADEPT) algorithm. A novel approach to the multivariate two-class events classification of sparse data in a multidimensional space

has been developed based on classification in multiple k-dimensional projections with subsequent applying a decision fusion algorithm to form a final classifier. The discrimination within a single k-dimensional projection was performed using a kernel based probability density estimator with adoptive bandwidth (ADE) by creating a separate density probability estimations for both classification events and then generating the logit score reflecting the probability of a given data point to fall into one of the two event classes. The resulting score was obtained as a weighted sum of scores over all selected projections. The optimal set of projections was then obtained by utilizing the projection pursuit technique (PT) (1) applied to the simulation set which was comprised of series of the training subsets created by repetitive random sub-sampling of the original data set and adding the Gaussian white noise to each data point as well as a scale noise to the whole training subset in the form of the linear transform with random coefficients in order to reflect the scale de-synchronization between successive multiplexed runs.

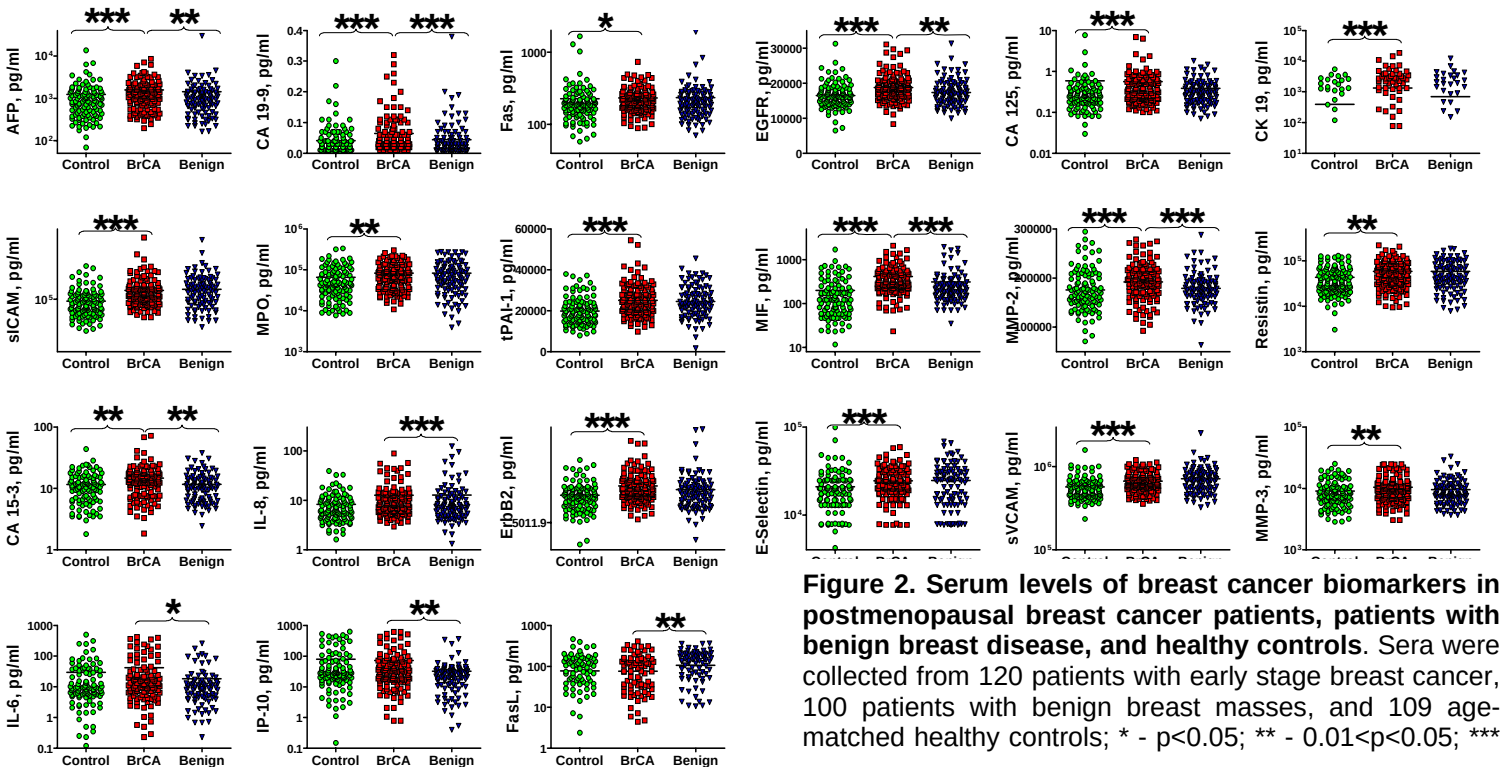


Figure 2. Serum levels of breast cancer biomarkers in postmenopausal breast cancer patients, patients with benign breast disease, and healthy controls. Sera were collected from 120 patients with early stage breast cancer, 100 patients with benign breast masses, and 109 age-matched healthy controls; * - p<0.05; ** - 0.01<p<0.05; ***

The data for 53 markers were then analyzed using ADEPT algorithm. A 23-biomarker panel was selected using the projection pursuit technique including IL-6, TNF-α, MCP-1, MIP-1α, MIP-1β, VEGF, bFGF, G-CSF, HGF, IP-10, TNFRI, IL-2R, IGFBP-I, AFP, mesothelin, CA 72-4, CEA, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM (listed in the order of their importance). The resulting model led to correctly classifying **84%** of the test set observations, with a sensitivity of **85%** at a specificity of **83%** or **75%** SN at **90%** SP (Figure 3). The classification power of the 23-biomarker panel is comparable with that of mammography for postmenopausal population. The discrimination of breast cancer from benign breast disease resulted in **65%** sensitivity at **75%** specificity. *These results indicate that combining multiple biomarkers results in higher diagnostic power for breast cancer than that offered by each individual biomarker.* Applying such a multimarker panel does not result in overfitting since the same panel demonstrated similar but 3-5% lower classification when logistic regression or classification trees algorithms were used (data not shown). *The classification power achieved in this study using the 23-biomarker panel in serum is not sufficient for screening of general population. However, the diagnostic power of this panel may be further improved by considering additional clinical information, such as ER/PR and Her2/neu status, Breast Imaging Reporting and Data System (BI-RADS) category, etc. It is also possible that this panel could diagnose women missed by mammography. All these studies will be performed in the proposed application.*

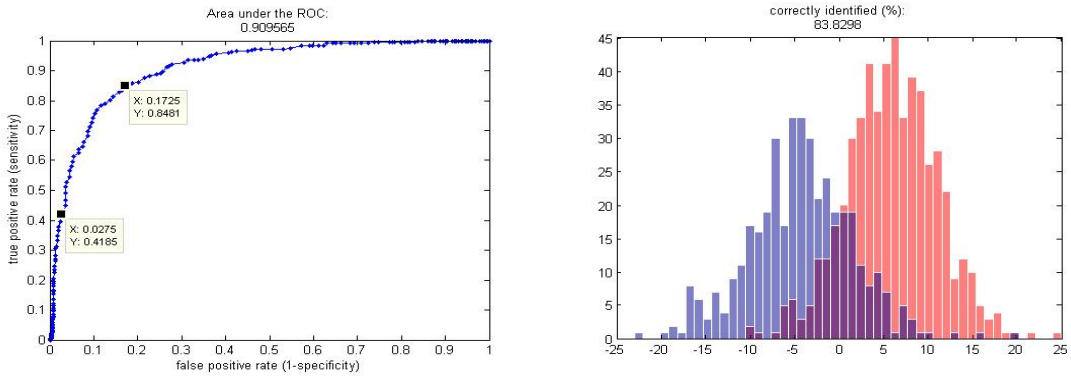


Figure 3. Cumulative ROC curve for postmenopausal breast cancer vs. healthy with 30 biomarkers panel using the ADEPT algorithm, 55/45 split. A. Cumulative ROC curve. 10-fold cross validation of patients with breast cancer (n=120) and healthy controls (n=109). Cross validation test, 55/45 random split, 100 runs (30-marker panel); **B. Cumulative histogram for ADE algorithm (55/45 split, 10 runs).** The results of the random split analysis displayed as a Cumulative histogram. Analysis was performed using ADE-PT algorithm, 55/45 random split (55% training set, 45% validation set).

Menopausal status-specific analysis of women with breast cancer. Next, the multi-biomarker analysis was performed using serum samples from post- (n=76) vs. premenopausal (n=34) patients with breast cancer. The data revealed that IL-8, Eotaxin, VEGF, IP-10, IGFBP-I, AFP, sFas, Cyfra 21-1, and sVCAM-1 levels were significantly higher in postmenopausal patients as compared to premenopausal (Figure 4). In contrast, concentration of CA 19-9 was significantly lower in postmenopausal women. *These results suggest that the expression of serum cytokines is different in these two tested groups. Furthermore, the data suggest that separate cytokine panels should be developed for breast cancer screening in women based on their menopausal status.*

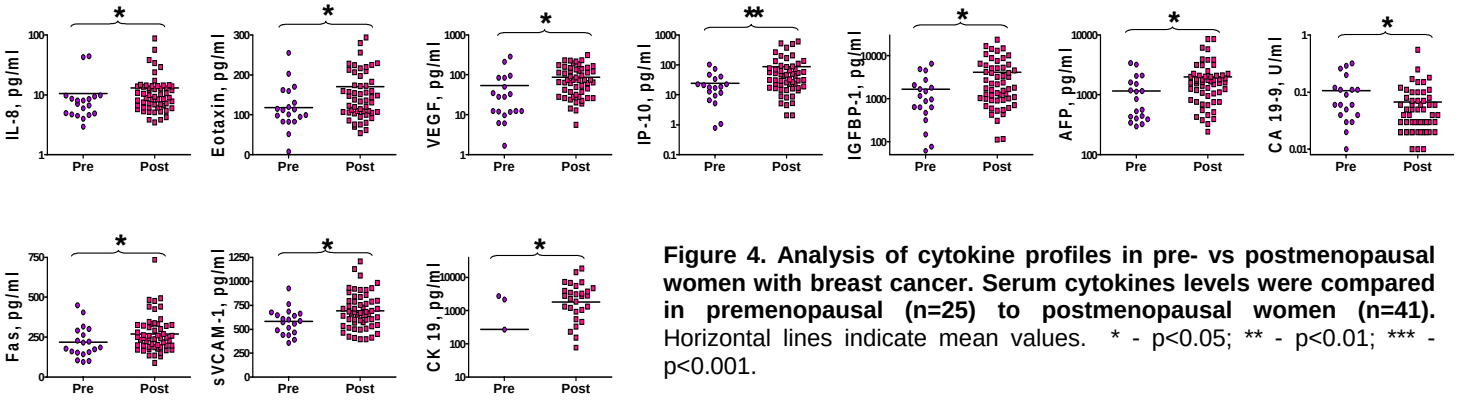


Figure 4. Analysis of cytokine profiles in pre- vs postmenopausal women with breast cancer. Serum cytokines levels were compared in premenopausal (n=25) to postmenopausal women (n=41). Horizontal lines indicate mean values. * - p<0.05; ** - p<0.01; *** - p<0.001.

Multimarker approach for early detection breast cancer in premenopausal women. Based on the above data we have performed similar analyses using sera from premenopausal women: 52 patients with breast cancer in comparison to 59 healthy women and 49 women with benign conditions. An expanded 87- marker bead-based multi-biomarker xMAP panel including 34 additional cancer-related analytes was utilized to screen sera of premenopausal patients with breast cancer and healthy controls. This panel included cancer antigens, CA-125, CA 15-3, CA 19-9, CEA, AFP, CA 72-4; growth/angiogenic factors, EGF, VEGF, bFGF, IGFBPI, HGF, ErbB2, EGFR; apoptosis-related molecules, Fas, FasL, Cyfra 21-1; metastasis-related molecules, MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, tPAI, PAI -1 (active), sICAM, sVCAM, sE-selectin; cytokines and their receptors, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, TNFR I, TNFR II, CD40L, TGFα, IFNα, IFNγ, GM-CSF, G-CSF, M-CSF, IL-1Rα, IL-2R, IL-6R, IP-10, MCP-1, MCP-2, MCP-3, MIP-1α, MIP-1β, MIF, MIG, eotaxin, RANTES; hormones, βHCG, FSH, LH, GH, TSH, prolactin; kallikreins 8 and 10, resistin, adiponectin, leptin, NGF, MICA, S-100, DR5, mesothelin, and myeloperoxidase (MPO), ULBP-1,2, S100, angiostatin. Our results have demonstrated that the expression of CA 125, EGF, ErbB2, AFP, FSH, CD40L, MMP-7, MIF, and PAI-I (active) was increased and SCC was decreased in

premenopausal women compare to healthy individuals (Figure 5). Furthermore, serum levels of IL-10, GM-CSF, CA 19-9, CA 125, EGF, and HGF were significantly higher in premenopausal women with breast cancer compare to benign disease group. In contrast, we observed lower expression of serum IL-4, IL-5, and IFN-γ in premenopausal breast cancer patients compare to patients with benign conditions (Figure 6).

Using the ADEPT algorithm, we have identified a 20-marker panel (MIF, MMP-2, Mesothelin, MIG, CA 125, sIL-6R, FSH, TSH, sFasL, EGF, prolactin, RANTES, ULBP-2, MCP-1, GH, ErbB2, SCC, MIP-1b, S100, ACTH) that provide a sensitivity of **88%** at a specificity of **89%** on cross-validation testing of breast cancer vs. healthy individuals (Figure 6), *which is substantially higher than that for mammography in this age group*. Furthermore, an independent 20-biomarker panel (TNFR-II, CA 125, IL-10, TNFR-II, G-CSF, HGF, MMP-1, IL-2, GH, MCP-3, MPO, CA 72-4, MMP-7, IL-12p40, CA 19-9, mesothelin, PAI-I (active), IL-6R, MIF, CEA) was able to identify premenopausal women with breast cancer vs. with benign conditions with **83%** sensitivity at **80%** specificity as shown at Figure 6.

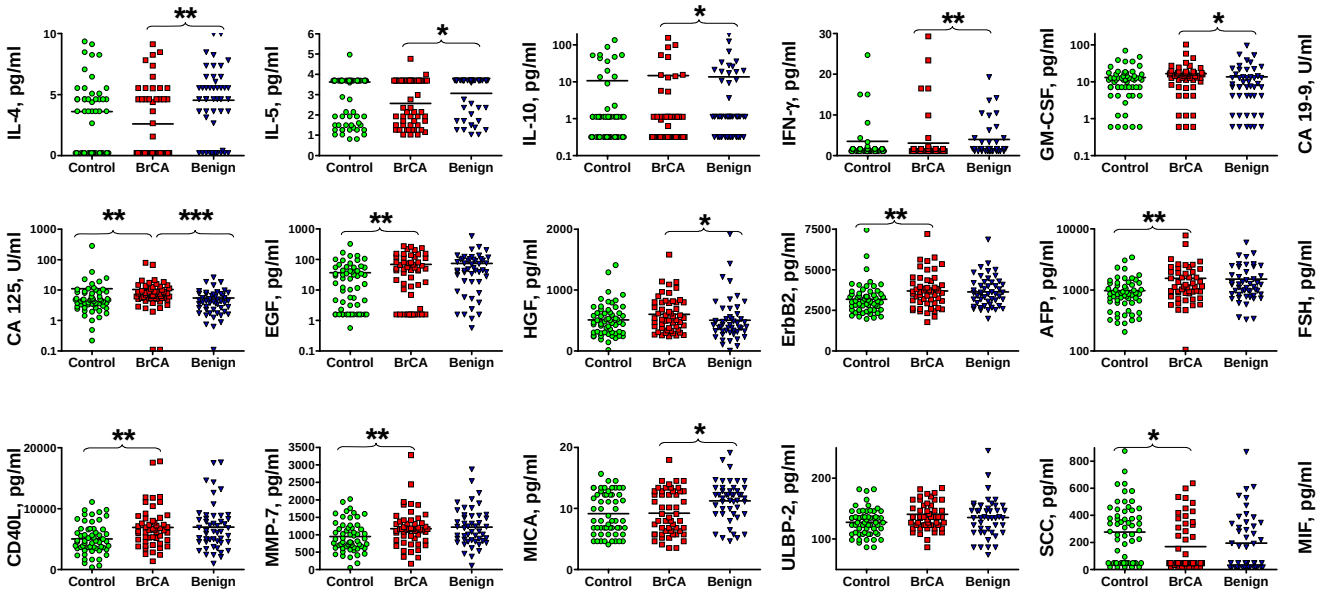


Figure 5. Serum levels of breast cancer biomarkers in premenopausal women with breast cancer, benign breast disease, and healthy controls. Sera were collected from 52 premenopausal patients with early stage breast cancer, 49 patients with benign breast masses, and 59 age-matched healthy controls. Circulating concentrations of the markers were measured using xMAP technology. Horizontal lines indicate mean values. * - $p<0.05$; ** - $0.01<p<0.05$; *** - $p<0.001$.

Taken together, our data suggest that multimarker approach allows for improved classification of breast cancer. Additionally, use of biomarkers belonging to different functional group may result in improving both specificity and sensitivity. High degree discrimination between cancer and control groups indicates that serum cytokine profiles could potentially be used for development of blood-based diagnostic test for early detection of breast cancer. However, while these markers have some power to discriminate between premenopausal women with breast cancer, benign disease, and healthy individuals, it is clear that additional markers are needed in order to improve diagnostic power.

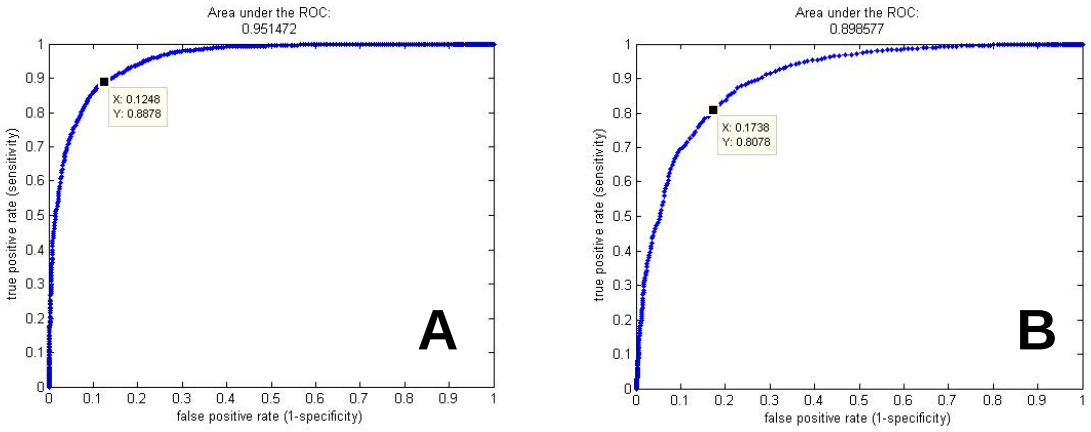


Figure 6. Cumulative ROC curve A. breast cancer vs. healthy controls; B. breast cancer vs benign disease with 2 distinguished 20-biomarker panels using ADEPT algorithm, 55/45 random split (55% training set, 45% validation set), 100 runs.

Task 4. Analyze the same serum samples using SELDI-TOF-MS technique. Construct a clinical prediction rule to identify SELDI-TOF-MS markers of breast cancer.

Mammogram information was obtained from patient's medical records under consent. If no mammogram data is available, patient will be assigned to BI-RADS 0. mammogram information was added to SELDI and other clinical data to develop CART decision tree models.

Results

SELDI with other clinical data (age, smoking, and drinking).

	Ca Vs Normal			Ca Vs Benign			Benign Vs Normal		
	Best Classifier	Top 10% Range	Median of All	Best Classifier	Top 10% Range	Median of All	Best Classifier	Top 10% Range	Median of All
Sensitivity	0.63	0.56~0.75	0.56	0.63	0.38~0.63	0.38	0.75	0.69~0.75	0.69
Specificity	0.63	0.5~0.63	0.5	0.81	0.75~0.88	0.88	0.81	0.81	0.81
Misclassification Rate	0.38	0.38~0.41	0.5	0.28	0.28~0.38	0.38	0.22	0.22~0.25	0.25

SELDI, clinical data, and mammogram BI-RADS category.

	Ca Vs Normal			Ca Vs Benign			Benign Vs Normal		
	Best Classifier	Top 10% Range	Median of All	Best Classifier	Top 10% Range	Median of All	Best Classifier	Top 10% Range	Median of All
Sensitivity	0.8	0.8	0.8	0.88	0.32~0.88	0.38	0.75	0.69~0.88	0.69

Specificity	0.82	0.82	0.82	0.81	0.5~0.88	0.88	0.94	0.75~0.94	0.81
Misclassification Rate	0.21	0.21	0.21	0.16	0.16~0.38	0.38	0.16	0.16~0.22	0.25

There is significant improvement in all three groups. The major change in Ca vs. Normal group is due to the clear-cut difference of mammogram between Ca and Normal. The features used in best decision tree are all BI-RADS categories. The most important is the better performance in Ca vs. Benign group, which will help in differentiating benign lesions and avoiding unnecessary biopsies.

In order to compare how SELDI and mammogram BI-RADS data affecting the CART decision tree performance, we used mammogram BI-RADS and other clinical data without SELDI data to develop decision tree models.

Performance of Testing set

	Ca Vs Normal			Ca Vs Benign			Benign Vs Normal		
	Best Classifier	Top 10% Range	Median of All	Best Classifier	Top 10% Range	Median of All	Best Classifier	Top 10% Range	Median of All
Sensitivity	0.8	0.8	0.8	0.88	0.88	0.63	0.94	0.94	0.94
Specificity	0.82	0.82	0.82	0.44	0.44	0.56	1	1	1
Misclassification Rate	0.21	0.21	0.21	0.34	0.34	0.46	0.03	0.03	0.03

It suggested that mammogram BI_RADS and other clinical data contributed mostly to the performance of Ca vs. Benign and Benign vs. Normal. But adding SELDI data can improve the specificity of Ca vs. Benign significantly.

In summary, SELDI and mammogram BI-RADS data all have their advantages in differentiating different group of breast lesions. Combination of both will improve overall performance.

Task 5. Combine the breast cancer-specific marker panels to identify an integrated marker panel with the highest predictive value.
This task is currently in progress. Once it is accomplished, the data will be transferred to DOD and reported to the scientific community. The estimated time-line is 1 month.

Task 6. Test the integrated assay for predictive value in a longitudinal fashion. This task is currently in progress. Once it is accomplished, the data will be transferred to DOD and reported to the scientific community. The estimated time-line is 3 years. The approach is described below:
We plan to next evaluate the final screening test in the *Prospective Screening Studies***.** In a prospective screening study, the assay is applied to target population, and individuals that screen positive undergo definitive diagnostic procedures. Therefore, studies at this stage involve screening people and lead to diagnosis and treatment. The aims of this phase include assessment of (i) the potential benefit incurred by early detection; and (ii) the effects of screening on costs and mortality associated with cancer. For this Phase, a standard parallel-arm randomized clinical trial will be undertaken in large population of subjects, with one arm consisting of subjects undergoing the screening protocol and the other arm consisting of unscreened subjects.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a multimarker panel for early diagnosis of breast cancer in postmenopausal women with 85% sensitivity and 83% specificity
- Developed a multimarker panel for early diagnosis of breast cancer in premenopausal women with 88% sensitivity and 89% specificity, which exceeds the power of mammography
- Identified proteomic pattern associated with breast cancer

REPORTABLE OUTCOMES:

Manuscripts:

Drukier AK, Ossetrova N, Schors E, Krasik G, Grigoriev I, Koenig C, Sulkowski M, Holcman J, Brown LR, Tomaszewski JE, Schnall MD, Sainsbury R**, Lokshin AE**, Godovac-Zimmermann J. High-Sensitivity Blood-Based Detection of Breast Cancer by Multi Photon Detection Diagnostic Proteomics. J Proteome Res. 2006 Aug 4;5(8):1906-1915.

Yurkovetsky Z, Lisovich A, Linkov F, Marrangoni A, Velikokhatnaya L, Nolen B, Winans M, Modugno F, Gorelik E, Marks J, **Lokshin A**. Multimarker approach for early diagnosis of breast cancer. Submitted to Breast Cancer Research.

Gorelik, E., Landsittel, DP, Marrangoni, AM, Modugno, F, Velikokhatnaya, L, Winans, MT, Bigbee, WL, Herberman, RB, **Lokshin, A**. Multiplexed Immunobead-Based Cytokine Profiling for Early Detection of Ovarian Cancer. Cancer Epidemiol Biomarkers Prev. 2005 Apr;14(4):981-7.

Gorelik E, Landsittel D, Winans M, Marrangoni AM, Velikokhatnaya L, **Lokshin AE**. IL-8 and anti-IL-8 antibody in ovarian cancer. Gynecol Oncol. 2006 102:244-251.

Yurkovetsky Z, Lisovich A, Skates S, Jacobs I, Menon U, Marrangoni A, Velikokhatnaya L, Nolen B, Winans M, Modugno F, Diamandis E, Gorelik E, Badgwel D, Bast RC, Jr, **Lokshin A**. Development of a multimarker assay for early detection of ovarian cancer. Under consideration for NEJM.

Abstracts:

Serum biomarker for early detection of breast cancer. DOD Era of Hope Meeting. June 2005

Circulating Markers for Breast Cancer Discrimination Assayed by Luminex. EDRN Steering Committee Meeting. March 2006.

Multiplexed approach for early detection of breast cancer. AACR Meeting, 2006

Multiplexed approach for early detection of breast cancer. 4th Annual Research Day University of Pittsburgh

Presentations:

Serum biomarker for early detection of breast cancer. DOD Era of Hope Meeting. June 2005

Serum biomarker for early detection of breast cancer. Maimonides Society, United Jewish Federation, Pittsburgh, 2006

Patents and licenses applied for and/or issued: Breast Cancer Assay. June 2006. Method and Composition for Diagnosing Breast Cancer. Provisional Patent Application 60-809960.

Funding applied for based on work supported by this award:

Susan Komen Foundation: Multiplexed biomarkers for early detection and remission monitoring of breast cancer. Anna Lokshin, PI.

Avon/NIH: Multiplexed Assay for Early Detection of Breast Cancer. Anna Lokshin, PI

United Jewish Federation of Pittsburgh Research Award. Early detection of breast cancer in high-risk group. Anna Lokshin, PI

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

Anna Lokshin, PhD
William Bigbee, PhD
Francesmary Modugno, PhD
Adele Marrangoni, BS
Lyudmila Velikokhatnaya, MS

CONCLUSION:

At the conclusion of the proposed Project, we have generated possibly the most complete existing list of serological breast cancer biomarkers Serum samples obtained from several unique repositories and ongoing studies have been screened using these multiplexed biomarker arrays. We have generated the highly predictive test for early preclinical detection of breast cancer in premenopausal population, where the diagnostic power of mammographic examination is insufficient. The involvement of key clinical investigators in this study allowed the Principal Investigator access to clinical and demographic data regarding the tissue and serum samples that would not otherwise be available. This additional clinical data may be extremely important in extending inferences from the experimental data.

The results have been submitted for Provisional Patent Application. University of Pittsburgh Medical Centers have licensed the technology from Dr. Lokshin and plan to initiate a clinical trial for validating of this test as a screening modality for premenopausal women as well as for monitoring the remission in breast cancer patients. Considering the capability of xMAP^{TM} technology, this trial can be feasibly completed in the course of 3-5 years.

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1. Drukier, A. K., Ossetrova, N., Schors, E., Krasik, G., Grigoriev, I., Koenig, C., Sulkowski, M., Holcman, J., Brown, L. R., Tomaszewski, J. E., Schnall, M. D., Sainsbury, R., Lokshin, A. E., and Godovac-Zimmermann, J. High-sensitivity blood-based detection of breast cancer by multi photon detection diagnostic proteomics. J Proteome Res, *5:* 1906-1915, 2006.
2. Lokshin, A. E., Winans, M., Landsittel, D., Marrangoni, A. M., Velikokhatnaya, L., Modugno, F., Nolen, B. M., and Gorelik, E. Circulating IL-8 and anti-IL-8 autoantibody in patients with ovarian cancer. Gynecol Oncol, *102:* 244-251, 2006.
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APPENDICES

MULTIMARKER APPROACH FOR EARLY DIAGNOSIS OF BREAST CANCER.

Yurkovetsky Z, Lisovich A, Linkov F, Marrangoni A, Velikokhatnaya L, Nolen B, Winans M, Modugno F, Gorelik E, Marks J, Lokshin A.

ABSTRACT

Breast cancer is one of the most common causes of cancer related deaths in women. Clinical outcome of breast cancer patients may be improved by early detection. We hypothesized that a panel of several biomarkers representing tumor-secreted and systemic host response proteins will provide superior diagnostic power than existing methodologies. To test this hypothesis, we utilized a novel multiplexing xMAP® technology (Luminex Corp., Austin, TX) for screening of various serum biomarkers in breast cancer patients. Concentrations of 53 biomarkers, Cancer antigens, CA 125, CEA, AFP, CA 72-4, CA 15-3; growth/angiogenic factors, VEGF, bFGF, IGFBPI, HGF ErbB2, EGFR; apoptosis-related molecules, Fas, FasL, Cyfra 21-1; metastasis-related molecules, MMP-2, MMP-3, tPAI, sICAM, sVCAM, sE-selectin; Cytokines, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, TNFR I, TNFR II, IFNγ, GM-CSF, G-CSF, M-CSF, IL-2R, IP-10, MCP-1, MIP-1α, MIP-1β, MIF, eotaxin, RANTES; hormones, bHCG; kallikreins 8 and 10, resistin, mesothelin, and myeloperoxidase (MPO), were measured in sera of 120 patients with early stage breast cancer, 100 women with benign breast masses, and 109 age-matched healthy controls. AFP, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM, MPO, tPAI, MIF, MMP-2, resistin, CA 15-3, IL-8, ErbB2, sE-Selectin, sVCAM, and MMP-3 were significantly higher in breast cancer patients as compared to healthy controls. Additionally, serum concentrations of IL-6, IP-10, AFP, CA 15-3, CA 19-9, EGFR, MMP-2, and MIF were significantly lower and sFasL was significantly higher in women with benign breast disease as compared to patients with breast cancer. The data for 53 markers were then analyzed using ADEPT algorithm. A 23-biomarker panel was selected using the projection pursuit technique including IL-6, TNF-α, MCP-1, MIP-1α, MIP-1β, VEGF, bFGF, G-CSF, HGF, IP-10, TNFRI, IL-2R, IGFBP-I, AFP,

mesothelin, CA 72-4, CEA, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM (listed in the order of their importance). The resulting model led to correctly classifying 84% of the test set observations, with a sensitivity of 85% at a specificity of 83% or 75% SN at 90% SP. The classification power of the 23-biomarker panel is comparable with that of mammography for mixed pre- and postmenopausal population. The discrimination of breast cancer from benign breast disease resulted in 65% sensitivity at 75% specificity. These results indicate that combining multiple biomarkers results in higher diagnostic power for breast cancer than that offered by each individual biomarker. In comparison with published evidence, the presented assay offers so far the best diagnostic power for early detection of breast cancer.

INTRODUCTION

Breast cancer is the second leading cause of cancer related deaths in American women. With one million new cases in the world each year, breast cancer is the most common malignancy in women (other than skin cancer) and constitutes 18% of all female cancers [1]. In the US breast cancer accounts for about 30% of all cancers diagnosed. The occurrence of breast cancer is usually suggested either by physical exam or as a result of screening mammography. The goal of screening mammography is to diagnose patients with breast cancer at an earlier stage, which significantly affects prognosis [2]. However, not all breast cancers are detected by screening mammography and not all cancers are identified at the ideal earliest stage at which cure can be expected [3-5]. In addition, still some 70% to 80% of breast biopsies from patients with an abnormal radiographic image demonstrate a benign process. This represents an enormous cost and generates considerable anxiety for the women with suspected breast cancer [6-8]. Various additional imaging methods including sonography, magnetic resonance tomography, thermography, and other techniques currently lack the requisite sensitivity and specificity [9, 10]. The ideal serological marker for malignancy of the breast has not yet been identified. While several markers are elevated in many patients with breast cancer, such as CA 15-3, MUC1 and HER-2/neu [11] any single

assay currently lacks the necessary sensitivity and specificity to be broadly useful as a primary screening tool. Therefore, there is a compelling need of a new assay, which would provide earlier, more sensitive and more specific detection of breast cancer.

Circulating tumor markers in patients affected by breast cancer have been known for several years. In contrast to markers detected in the primary tumor, they offer dynamic information regarding the clinical evolution of the neoplastic process. Currently, no tumor marker exists that can be used for either screening or the early diagnosis of breast cancer. In fact, the diagnostic accuracy of tumor marker evaluation is limited by low sensitivity in early-stage disease and by lack of specificity. Regarding CA 15-3, for instance, different studies have demonstrated that the diagnostic sensitivity of the test is about 10–15%, 20–25% and 30–45% in patients with stage I, stage II and stage III disease, respectively (reviewed in [12]). Furthermore, increased levels of CA 15-3 can be observed in several non-neoplastic conditions, including benign breast pathology, chronic liver disorders and immunological diseases. Likewise, several other biomarkers identified in serum of breast cancer patients including CEA, CA 15-3, TPA/TPS, Cyfra 21-1, CA 125, HGF, IL-6, VEGF, CA72-4, kallikreins 6,10, etc., taken singly, do not provide sufficient diagnostic power for this disease [13-21].

Recent research suggested that mammaglobin A protein could be used in breast cancer diagnosis and treatment. Mammaglobin A (SCGB2A2), the member of the secretoglobin superfamily, is known to be expressed in breast cancer [22]. Studies suggest that mammaglobin is one of the first relatively mammary-specific and mammary-sensitive markers (85%). Mammaglobin may be valuable when used in a panel with BRST-2 (GCDFP-15) and ER for evaluating tumors of unknown primary sites (Antibody Datasheet http://www.biocare.net/AntibodyView.asp?ID=528). In this study, our group would like to harness the efficacy of mammaglobin A in breast cancer screening and evaluate it as a part of our new panel. Recently, several ovarian cancer studies reported that combining information on several biomarkers using flexible statistical methods may result in improved diagnostic power of such multimarker assays [23, 24].

In the present study, we have utilized a multiplexed panel consisting of 53 proteins associated with epithelial cancers to identify a combination of biomarkers with the highest discriminating power for detection of early stage breast cancer. We utilized a novel multiplexing xMAP® technology (Luminex Corp., Austin, TX) and the adaptive bandwidth kernel based density estimator combined with projection pursuit technique (ADEPT) to create a diagnostic model based on the cross-validation set consisting of sera from 120 patients with early stage breast cancer, 100 women with benign breast masses, and 109 age-matched healthy women.

PATIENT POPULATIONS AND METHODS

Patient populations. The serum samples from 120 patients with early stage breast cancer, 100 women with benign breast masses, and 109 age-matched healthy controls age-matched controls were tested. Serum samples from patients with breast cancer, women with benign disease, and healthy women were provided by Dr. Jeffrey Marks (Duke University, Dunham, SC). Patients were enrolled under their IRB protocols. Information about diagnoses and breast cancer staging as well cancer histology and grade was provided by the Duke University. No data allowing identification of patients were provided. All major types of breast cancer and a variety of benign conditions were represented in these series (Table I). Additional control serum samples from healthy, age-matched women were received from Dr. Francesmary Modugno (UPCI, Pittsburgh, PA). Written informed consent was obtained from each subject or from his or her guardian. Sample collection was performed after approval by the institutional review board and in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services.

Collection and storage of blood serum. Peripheral blood samples were collected following informed consent using standard venipuncture techniques into sterile 10 ml BD Vacutainer™ glass serum (red top) tubes (BD, Franklin Lakes, NJ). All blood samples were encoded at the time of collection with a

unique study alphanumeric identifier linked in a separate and secure datafile to the subject's name and social security number, together with the date and time of collection, to ensure subject confidentiality and to blind laboratory personnel. For processing, the red top tube was spun at room temperature at 20 x 100 rpm for 10 minutes in a Sorvall benchtop centrifuge. The serum fraction was then collected by pipetting into a pre-chilled tube on ice and mixed to ensure homogeneity of the serum sample. The serum was then divided into 1.0 ml aliquots. No significant differences were found in prior studies regarding biomarkers obtained on the first vs. a repeat aspiration visit. Processing time from sample collection to freezing at was within 4 h. The serum aliquots were stored at –80^{o}C or below. The serum samples were shiped for xMAP® analysis on dry ice to ensure the biological integrity of the samples. No more than 2 freeze-thaw cycles were allowed for each sample.

xMAP® bead-based assays. Fifty three xMap assays against most known breast cancer serum biomarkers were utilized in this study. xMAP assays for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, TNFR I, TNFR II, IFNγ, GM-CSF, G-CSF, M-CSF, IL-2R, IP-10, MCP-1, MIP-1α, MIP-1β, eotaxin, and RANTES were purchased from BioSource International (Camarillo, CA); assays for MMP-2 and -3 were from R&D Systems (Minneapolis, MN), assays for sICAM, sVCAM, sE-selectin, total plasminogen activator inhibitor-1 (tPAI-1), Resistin, and MIF were obtained from Linco Research (St. Charles, MO). Assays for CA 125, EGFR, HGF, VEGF, βFGF, ErbB2, CEA, CA 15-3, CA 72-4, AFP, IGFBP-1, HCG, Kallikreins-8,10, Cyfra 21-1, sFas, sFasL, MPO, and mesothelin were developed in Luminex Core Facility of University of Pittsburgh Cancer Institute according to the protocol by Luminex Corp. (Austin, TX) essentially as described earlier for CA 125 [25]. Of these, antibody pairs for kallikreins were provided by Dr. Diamandis (Malvern, PA). Chicken polyclonal antibody for human full-length mesothelin was developed by GenWay Biotech, Inc. (San Diego, CA). Overall, 7 different multiplexed panels were used. The minimum detection levels for representative antigens are presented in Table II. Inter-assay variability, expressed as a coefficient of

variation, was calculated based on the average for ten patient samples and standards that were measured in four separate assays. The inter-assay variability within the replicates presented as an average coefficient of variation was in the range of 3.2 to 7.1% (Table II). Intra-assay variability was evaluated by testing quadruplicates of each standard and ten samples measured three times. The variabilities of these samples were between 5.1 and 8.6% (Table II). Each assay was further validated in comparison with appropriate ELISA and has demonstrated 97-99% correlation (Table II).

Multiplex analysis. The xMap serum assays were performed in 96-well microplate format as previously described [25]. All purchased assays were performed according to appropriate manufacturer's protocols. In-house assays were performed as previously described for CA 125 using serum dilution of 1:5 [25]. Samples were analyzed using the Bio-Plex suspension array system, (Bio-Rad Laboratories, Hercules, CA). Analysis of experimental data was performed using five-parametric-curve fitting.

Statistical analysis of data. Descriptive statistics and graphical displays (i.e. dot plots) were prepared to show the distribution of each marker for each disease state with Graphpad Prism version 2.0 (Graphpad Software, Inc., San Diego, CA). The Kruskal-Wallis One Way Analysis of Variance on Ranks was used to evaluate the significance of differences in marker expression between each disease state. All Pairwise Multiple Comparison Procedures (Dunn's Method) was also incorporated to quantify the relationships between groups for each marker. An adaptive bandwidth kernel based density estimator combined with projection pursuit technique (ADEPT), a novel approach to the multivariate two-class events classification of sparse data in a multidimensional space has been developed as previously described [26]. The level of significance was taken as $P<0.05$.

RESULTS

xMAP®-based analysis of serum concentrations of different biomarkers in breast cancer patients. Fifty three xMap assays against most known ovarian cancer serum biomarkers were utilized in patients with breast cancer and healthy controls. Those included cancer antigens, Cancer antigens, CA 125, CEA,

AFP, CA 72-4, CA 15-3; growth/angiogenic factors, VEGF, bFGF, IGFBPI, HGF ErbB2, EGFR; apoptosis-related molecules, Fas, FasL, Cyfra 21-1; metastasis-related molecules, MMP-2, MMP-3, tPAI, sICAM, sVCAM, sE-selectin; Cytokines, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, TNFR I, TNFR II, IFNγ, GM-CSF, G-CSF, M-CSF, IL-2R, RANTES, IP-10, MCP-1, MIP-1α, MIP-1β, MIF, eotaxin; hormones, βHCG; hK 8, hK 10, resistin, mesothelin, and MPO. Circulating concentrations of these proteins were evaluated in a multiplex assay using xMAP® technology, in serum samples of patients from 120 women with breast cancer and 109 age-matched healthy controls. Serum levels of VEGF, bFGF, IGFBPI, HGF, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, TNFR I, TNFR II, IFNγ, GM-CSF, G-CSF, M-CSF, IL-2R, MCP-1, MIP-1α, MIP-1β, eotaxin, RANTES, sFas, βHCG, hK 8, hK 10, and mesothelin were detectable but not statistically different in all tested clinical groups. Serum concentrations of AFP, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM, MPO, tPAI, MIF, MMP-2, resistin, CA 15-3, IL-8, ErbB2, sE-Selectin, sVCAM, and MMP-3 were significantly elevated in patients with breast cancer as compared to healthy controls ($p<0.05$ – $p<0.001$; Figure 1).

Next, the group with benign disease was compared to breast cancer group. Serum concentrations of IL-6, IP-10, AFP, CA 15-3, CA 19-9, EGFR, MMP-2, and MIF were significantly lower and sFasL was significantly higher in women with benign breast disease as compared to patients with breast cancer ($p<0.05$ - $p<0.001$; Figure 1). Therefore, potential biomarkers have a significantly different expression patterns in the in serum samples of patients from women with early stage breast cancer, women with benign masses, and age-matched healthy controls.

Statistical analysis of multimarker panels. None of the individual markers had a high enough sensitivity (1%-55%) at 98% specificity (Table III) to serve as a maker for early diagnosis. Multimarker analysis using ADE algorithm (part of ADEPT) was performed using set of 12 markers offering the best separation t-value. Data set consisting of 120 breast cancer samples and 109 control samples was randomly split into the training and validation sets at 55/45 ratio, then the training set was used to build

the classification model and the validation set was used to determine the total number of classification errors as well as the specificity/sensitivity. The described procedure was repeated 20 times for each algorithm and the average error rate and the cumulative specificity/sensitivity dependency were calculated.

In the following step, data for 53 markers were analyzed using ADEPT algorithm. A 23-biomarker panel was selected using the projection pursuit technique including IL-6, TNF-α, MCP-1, MIP-1α, MIP-1β, VEGF, bFGF, G-CSF, HGF, IP-10, TNFRI, IL-2R, IGFBP-I, AFP, mesothelin, CA 72-4, CEA, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM (listed in the order of their importance). The resulting model led to correctly classifying 84% of the test set observations, with a sensitivity of 84% at a specificity of 83% or 42% SN at 98% SP (Figure 2). The ROC curve representing the results of classification based on these biomarkers is presented on Figure 2A.

Classification of breast cancer vs. benign disease. Classification of benign vs. breast cancer group was performed using 120 sera from breast cancer patients and 100 sera from patients with benign masses as described above for discrimination of breast cancers from healthy controls. Projection pursuit algorithm identified 30 biomarkers for optimal discrimination, TNFα, MIF, ErbB2, MMP-2, CA 15-3, sFas, TNFRI, sE-Selectin, CA 72-4, CA 19-9, AFP, ICAM, IL-2R, HGF, EGFR, MCP-1, G-CSF, MIP-1α, VEGF, IL-8, VCAM, Mesothelin, IL-6, βFGF, MPO, CA-125, CA 15-3, Cyfra 21-1, IP-10, and HCG (listed in order of classification power). The discrimination of breast cancer from benign breast disease resulted in 70% sensitivity at 80% specificity or 26% sensitivity at 97% specificity with 75% correctly identified (Figure 3). These results indicate that combining multiple biomarkers results in higher diagnostic power for breast cancer than that offered by each individual biomarker. Applying such a multimarker panel does not result in overfitting since the same panel demonstrated similar but 3-5% lower classification when logistic regression or classification trees algorithms were used (data not shown). The higher threshold for specificity was chosen to reduce the number of ovarian cancer samples falling into the benign group.

Histology-specific analysis of women with breast cancer. The ability to separate breast cancer by histological types has a great potential for better therapy. To examine different tumor types, we analyzed circulating biomarker concentrations breast tumors with diverse histologies. We separated breast cancer samples into 5 groups: 23 Invasive Ductal, 13 Infiltrating Ductal, 11 Ductal Carcinoma in Situ (DCIS), 30 Invasive Infiltrating Ductal, and 10 Lobular. Serum concentrations of Eotaxin, IP-10, TNFR-I, hK10, Mesothelin, CA 125, and MPO demonstrated statistically significant differences between different histological groups of breast cancers ($0.001<p<0.05$; Figure 4). These results suggested that breast cancer patients have different biomarker profiles when separated by tumor types. Therefore, development of separate cancer biomarker panels for different histology groups of breast cancer may help selecting more appropriate treatments.

Menopausal status-specific analysis of women with breast cancer. Next, patients with breast cancer were analyzed based on their menopausal status. Women with breast cancer were separated into two groups: pre- and postmenopausal, and analyzed separately. The data revealed that IL-8, Eotaxin, VEGF, IP-10, IGFBP-I, AFP, sFas, Cyfra 21-1, and sVCAM-1 levels were significantly higher in postmenopausal patients as compared to premenopausal (Figure 5). In contrast, concentration of CA 19-9 was significantly lower in postmenopausal women. These results suggest that the expression of serum cytokines vary in these two tested groups. Furthermore, the data may suggest that separate cytokine panels should be developed for breast cancer screening in women based on their menopausal status.

DISSCUSION

We have demonstrated that multiplexing has led to the identification of a new grouping of biomarkers that could possess high diagnostic power for breast cancers with 83% specificity and 84% sensitivity. The new grouping of biomarkers is comprised of known breast, and other cancer markers: EGFR (breast and ovarian cancers), CA 125 (ovarian cancer), CA19-9 (gastric carcinoma, lung carcinoma, hepatoma, pancreatic carcinoma and ovarian cancer), AFP (hepatocellular carcinoma), CEA (colorectal cancer), and

Cyfra 21-1 (lung and ovarian cancers). Therefore, the present study revealed no unexpected links between known biomarkers and breast cancer.

In this study, we tested the possibility that a multimarker combination could possess high diagnostic power for breast cancers. The resulting panel consisted of 53 biomarkers, representing the largest multimarker panel ever analyzed in the same serum set. In agreement with published observations [11, 27-37], we observed elevated levels of AFP, CA 19-9, sFas, EGFR, CA 125, Cyfra 21-1, sICAM-1, MMP-2CA 15-3, IL-8, ErbB2, sE-Selectin, and sVCAM-1 in patients with breast cancer as compared with healthy women. Concentrations measured by xMAP® technology were comparable to previously reported studies that used ELISA assay. To the best of our knowledge, we were the first to report the elevated serum levels of MPO, tPAI, MIF, resistin, and MMP-3 in patients with breast cancer.

In this study, we observed that circulating levels of the apoptosis-related proteins sFas, sFas ligand, and HER2 were higher in breast cancer patients. The Fas/Fas ligand (FasL) system plays an important role in cellular apoptosis and is involved in cancer cell death induced by the immune system and anticancer drugs. Increased serum level of soluble Fas (sFas) is associated with a number of different disease states and with tumor progression and metastasis in patients. It has been suggested that circulating soluble levels may reflect the severity of invasive breast cancer [28]. Overexpression of the c-erbB2 gene is correlated with poor prognosis and the number of lymph node metastases in breast cancer patients [38]. The higher serum HER2 concentrations may be related to an altered basal apoptotic state. Several studies have linked HER2 to protection from apoptosis. For example, in ovarian cancer cells endogenous HER2 overexpression is associated with resistance to TNF-induced cytotoxicity [39]. The observed increased sFas and HER2 levels might suggest an interaction between HER2 shedding and cleavage of ligands and receptors involved in the death receptor apoptotic pathways.

The processes of cellular adhesion, migration, extracellular matrix degradation, invasion, proliferation, and neovascularization are influenced by numerous regulatory molecules found within the tumor microenvironment. These include EGFR, ErbB2, MMP-2, MMP-3, E-Selectin, VCAM, ICAM, and

cytokines such as IL-8. Many of these factors regulate the expression of the others and initiate a cascade of extracellular and intracellular signaling that stimulates hematogenous, lymphatic, and intraperitoneal metastatic dissemination. Specifically, an autocrine role of IL-8 modulating survival and proliferation of tumor cells has been suggested [40, 41]. Additionally, IL-8 was primarily known to be chemotactic for neutrophils. Inflammatory infiltrates have been associated with enhanced tumor growth and worse survival [42]. This might be attributed to the release of angiogenic growth factors by neutrophils and macrophages [43].

There are several different types of adenocarcinoma of the breast. Ability to detect these cancers early and being able to separate them by histological types has a great potential to provide better and timelier therapy for breast cancer patients. Ductal cancers represent the majority of invasive breast cancers (those with the potential to metastasize). The other common breast carcinoma is lobular. About 10 - 15% of breast carcinomas are infiltrating lobular. They present much more frequently as a mass and less commonly on mammograms than other types. There are several other types of invasive cancer which are much less common, have distinctive histologic features and tend to have a better outcome than the invasive ductal carcinomas. Lobular carcinoma is more difficult to detect than ductal carcinoma by both physical examination and mammography [44]. Therefore additional detection methods are needed to provide a substantially higher degree of specificity than presently afforded by screening mammography, especially for younger women and those at high risk of developing breast cancer. Our results suggest that different histological types of breast cancer should be evaluated separately because they have different serum cytokine profiles. Proper histologic categorization of breast carcinomas has significant prognostic implications [45]. Therefore, presently we are working on identification of biomarkers panels for early detection of breast cancer divided into several groups according to tumor histology.

The goal of screening mammography is to diagnose patients with breast cancer at an early stage, which significantly affects prognosis. However, not all breast cancers are detected by screening

mammography and not all cancers are identified at the ideal earliest stage at which cure can be expected. Overall sensitivity of mammography is approximately 75% [46] but ranges from 54% to 58% in women younger than 40 years to 81% to 94% in those older than 65 years at a specificity of 90% [47]. Mammography misses approximately 10% of early cancers in postmenopausal women, and up to 40% of cancers in premenopausal women [4, 5, 48]. Our data have demonstrated that pre- and postmenopausal women have different serum cytokine profiles. Therefore, the development of separate biomarker panels for these two groups of women may improve sensitivity and specificity of biomarker panels for early detection of breast cancer.

The blood test detects the presence of breast cancer at the molecular level and could offer advantages over mammography, which tends to only discover breast cancer after a mass has formed. Additionally, as a blood diagnostic procedure, the blood test is easier, safer and more cost-effective for healthcare professionals to administer than the existing mammography or MRI diagnostic procedures. In years to come, a serum test for every phase of cancer may drive clinical decision making, supplementing or replacing currently existing invasive techniques [49]. Our data demonstrate that a 23-biomarker panel, selected by the projection pursuit technique, led to correctly classifying 84% of the test set observations, with a sensitivity of 84% at a specificity of 83%. Thus, this panel may provide high classification power for detecting early stage disease at conventional diagnosis.

In conclusion, our group identified a panel of biomarkers that could be harnessed for the development of breast cancer screening test. Increasing our understanding of the role of biomarkers in the etiology and progression of breast cancer has a great potential to facilitate the development of new treatment modalities for this challenging disease.

Table I. Patient Characteristics

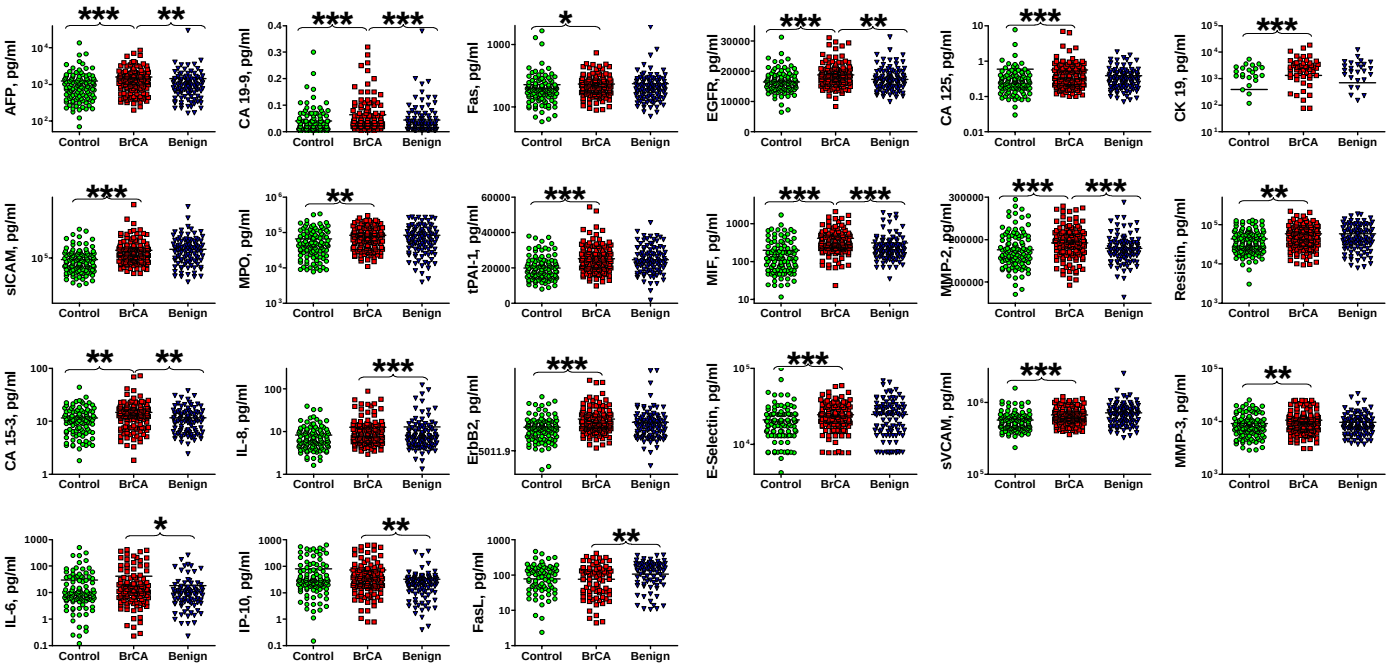
Patient Group	Age	Histology
Control	N=109 Range 22-84 Median 57 Average 55.2	
Breast Cancer	N=120 Range 20-82 Median 60 Average 59.5	Invasive ductal (n=28) Invasive lobular (n=5) Invasive metaplastic (n=1) Invasive tubular (n=2) Infiltrating ductal (n=14) Infiltrating lobular (n=3) Infiltrating mucinous (n=2) DCIS (n=23) Bilateral cancer (n=4) Invasive infiltrating Ductal (n=49)
Benign Tumors	N=100 Range 21-84 Median 55 Average 54.5	Fibrosis (n=3) Fibroadenoma (n=13) Fibrocystic changes (n=14) Benign breast tissue (n=5) Lipoma (n=3) Introductal papilloma (n=6) Introductal hyperplasia (n=4) Cyst (n=5) Others (n=46)

Table II. Validation of multiplexed xMAP® assays developed in UPCI Luminex Core Facility

Biomarkers	**CA 19-9**	**CA 125**	**CEA**	**CA 15-3**	**ErbB2**	**EGFR**	**Fas**	**FasL**	**Cyfra 21**
Sensitivity	20 IU/ml	5 U/ml	50 U/ml	1 IU/ml	50 pg/ml	5 pg/ml	15 pg/ml	50 pg/ml	300 pg/ml
Recovery (serum), %	96	101	99	98	100	98	98	95	89
Intra-assay variation, %CV	8.6	6.0	8.2	7.9	6.5	7.1	5.5	6.2	5.1
Inter-assay variation (%CV)	5.4	6.2	6.4	5.1	7.1	6.8	6.1	3.2	5.3
Correlation with ELISA	0.97	0.98	0.98	0.97	0.99	0.98	0.99	0.99	0.98

Table III. Breast cancer serum markers

Markers	Control	Cancer	p-value	Benign	p-value
IL-6		41.711±7.56	ns	18.237±3.80	p=0.047
IL-8	8.326±0.61	12.707±1.15	p<0.001		ns
IP-10	80.580±12.68	71.966±11.19	ns	32.789±5.64	p=0.008
AFP	1241.973±158.03	1588.510±124.92	P<0.001	1432.162±307.47	p=0.007
CA 15-3	11.586±0.67	14.783±0.93	P=0.004	11.745±0.69	p=0.007
CA 19-9	0.041±0.00	0.065±0.01	P<0.001	0.044±0.01	P<0.001
ErbB2	8343.536±216.52	10306.446±513.27	P<0.001	9297.341±382.847	ns
Fas	228.215±20.285	233.249±9.099	P=0.04	234.82±20.65	ns
EGFR	16495.696±349.24	18813.638±357.066	P<0.001	17363.308±374.377	P=0.003
CA 125	0.592±0.215	0.567±0.081	p<0.001	0.389±0.0309	ns
CK 19	394.852±94.376	1344.132±254.91	p<0.001	704.133±178.135	ns
FasL	77.778±8.768	76.651±11.848	ns	106.362±9.75	P=0.006
sE-Selectin	20974.258±1242.54	24436.977±903.99	p<0.001	24587.47±1377.129	ns
sVCAM	571586.99±18381.79	670549.83±16900.43	p<0.001	717364.855±29969.03	ns
sICAM	95499.024±3092.28	122191.458±4268.35	p<0.001	125559.129±5231.825	ns
MPO	65339.939±5851.18	83074.16±5440.556	P=0.011	83230.85±7042.00	ns
tPAI-1	19903.02±641.428	25135.665±721.11	p<0.001	24583.536±787.02	ns
MIF	198.67±23.096	408.96±31.19	p<0.001	309.669±35.296	p<0.001
MMP-2	175658.816±4115.548	192495.046±3720.185	p<0.001	179551.75±3647.717	P=0.003
MMP-3	9747.597±951.10	10472.975±478.14	P=0.019	9601.091±513.46	ns
Resistin	43184.955±2677.879	58021.07±3690.84	P=0.002	58479.623±4085.547	ns


AFP, pg/ml
CA 19-9, pg/ml
Fas, pg/ml
EGFR, pg/ml
CA 125, pg/ml
CK 19, pg/ml
sICAM, pg/ml
MPO, pg/ml
tPAI-1, pg/ml
MIF, pg/ml
MMP-2, pg/ml
Resistin, pg/ml
CA 15-3, pg/ml
IL-8, pg/ml
ErbB2, pg/ml
E-Selectin, pg/ml
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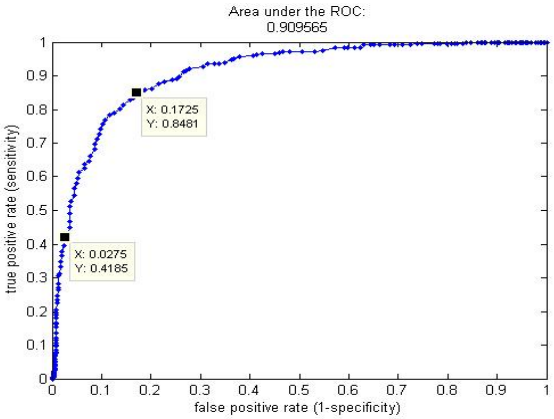
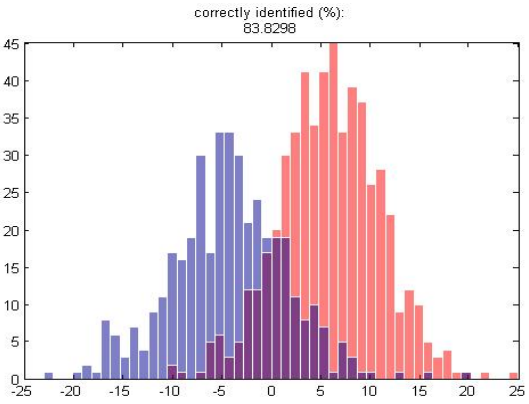
IP-10, pg/ml
FasL, pg/ml
Control
BrCA
Benign


Area under the ROC:
0.909565
X: 0.1725
Y: 0.8481
X: 0.0275
Y: 0.4185
true positive rate (sensitivity)
false positive rate (1-specificity)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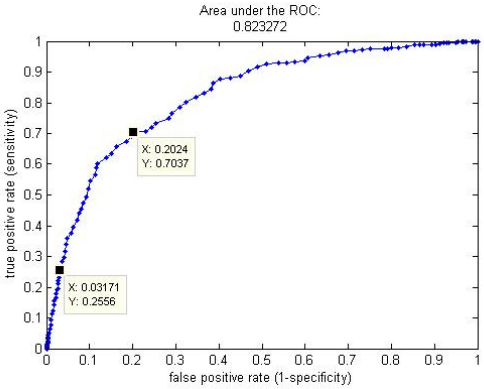
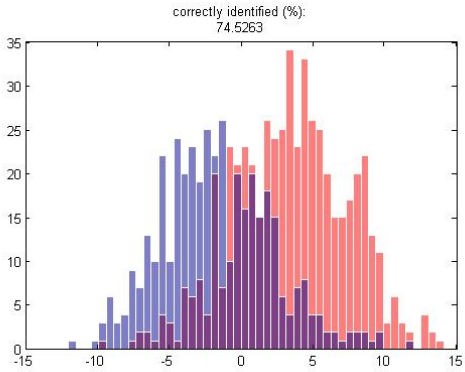

Area under the ROC:
0.823272
true positive rate (sensitivity)
false positive rate (1-specificity)
X: 0.2024
Y: 0.7037
X: 0.03171
Y: 0.2556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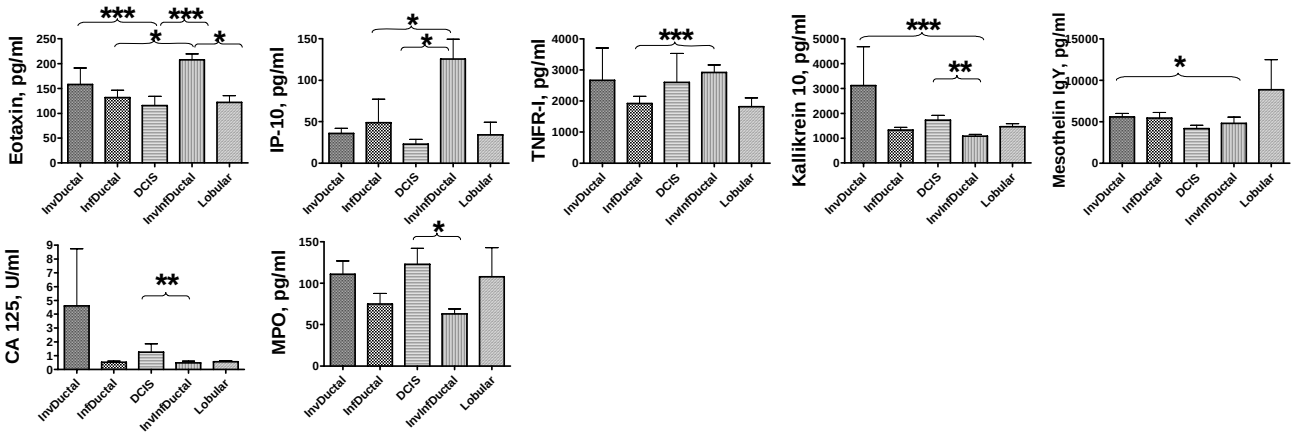
Eotaxin, pg/ml
IP-10, pg/ml
TNFR-I, pg/ml
Kallikrein 10, pg/ml
Mesothelin IgY, pg/ml
CA 125, U/ml
MPO, pg/ml
InvDuctal
InfDuctal
DCIS
InvInfDuctal
Lobular

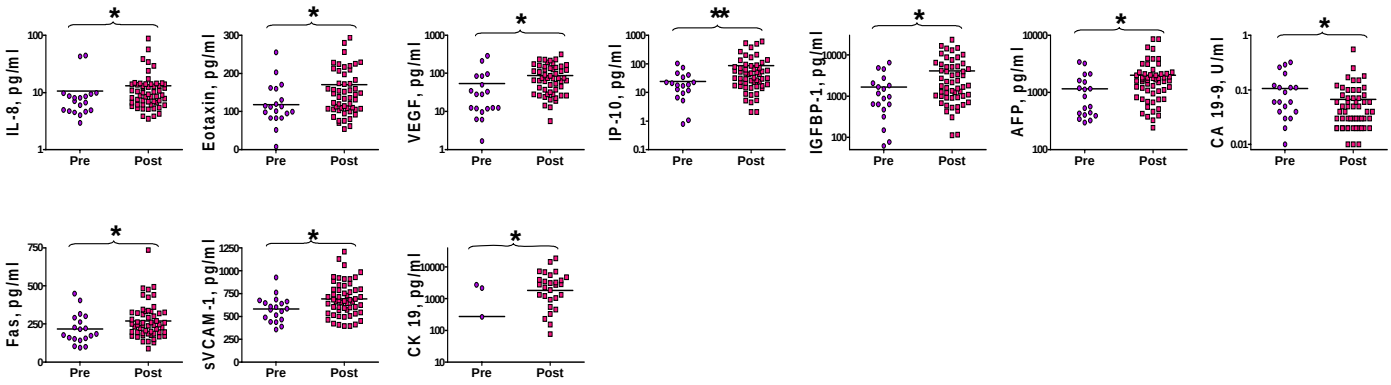
IL-8, pg/ml
Eotaxin, pg/ml
VEGF, pg/ml
IP-10, pg/ml
IGFBP-1, pg/ml
AFP, pg/ml
CA 19-9, U/ml
Fas, pg/ml
sVCAM-1, pg/ml
CK 19, pg/ml
Pre
Post
*
**

FIGURE LEGENDS

Figure 1. Serum levels of breast cancer biomarkers in healthy controls, breast cancer patients and patients with benign tumors. Sera were collected from 120 patients with breast cancer, 109 patients with benign masses, and from 100 age-matched healthy women. Circulating concentrations of markers were measured using xMAP® technology as described in Methods. Representative of two experiments is shown. Horizontal lines indicate mean values. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

Figure 2. Cumulative ROC curve for breast cancer vs. healthy with 23 biomarkers panel using the ADE-PT algorithm, 55/45 split. A. Cumulative ROC curve. 10-fold cross validation of patients with breast cancer (n=120) and healthy controls (n=109). Cross validation test, 55/45 random split, 100 runs (30-marker panel); **B. Cumulative histogram for ADE algorithm (55/45 split, 10 runs).** The results of the random split analysis displayed as a Cumulative histogram. Analysis was performed using ADE-PT algorithm, 55/45 random split (55% training set, 45% validation set).

Figure 3. Cumulative ROC curve for breast cancer vs. benign with 30 biomarkers panel using the ADE-PT algorithm, 55/45 split. A. Cumulative ROC curve. 10-fold cross validation of patients with breast cancer (n=120) and benign conditions (n=100). Cross validation test, 55/45 random split, 100 runs (30-marker panel); **B. Cumulative histogram for ADE algorithm (55/45 split, 10 runs).** The results of the random split analysis displayed as a Cumulative histogram. Analysis was performed using ADE-PT algorithm, 55/45 random split (55% training set, 45% validation set).

Figure 4. Comparative analysis of different histological types of breast cancer. Comparison between different histological types of breast cancer: Invasive Ductal (n=23), Infiltrating Ductal (n=13), DCIS (n=11), Invasive Infiltrating Ductal (30), and Lobular (n=10). * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

Figure 5. Analysis of cytokine profiles in pre- vs postmenopausal women with breast cancer. Serum cytokines levels were compared in premenopausal (n=25) to postmenopausal women (n=41). Horizontal lines indicate mean values. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

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Short Communication

Multiplexed Immunobead-Based Cytokine Profiling for Early Detection of Ovarian Cancer

Elieser Gorelik,[1,5] Douglas P. Landsittel,[2] Adele M. Marrangoni,[5] Francesmary Modugno,[3,5] Lyudmila Velikokhatnaya,[5] Matthew T. Winans,[5] William L. Bigbee,[5] Ronald B. Herberman,[4,5] and Anna E. Lokshin[4,5]

Departments of [1]Pathology and Immunology, [2]Biostatistics, and [3]Epidemiology, [4]Division of Hematology/Oncology, [5]University of Pittsburgh School of Medicine and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract

Early detection of ovarian cancer might improve clinical outcome. Some studies have shown the role of cytokines as a new group of tumor markers for ovarian cancer. We hypothesized that a panel comprised of multiple cytokines, which individually may not show strong correlation with the disease, might provide higher diagnostic power. To evaluate the diagnostic utility of cytokine panel, we used a novel multianalyte LabMAP profiling technology that allows simultaneous measurement of multiple markers. Concentrations of 24 cytokines (cytokines/chemokines, growth, and angiogenic factors) in combination with cancer antigen-125 (CA-125), were measured in sera of 44 patients with early-stage ovarian cancer, 45 healthy women, and 37 patients with benign pelvic tumors. Six markers, i.e., interleukin (IL)-6, IL-8, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), and CA-125, showed significant differences in serum concentrations between ovarian cancer and control groups. Out of this group, IL-6, IL-8, VEGF, EGF, and CA-125, were used in a classification tree analysis that resulted in 84% sensitivity at 95% specificity. The receiver operator characteristic curve created using the combination of markers produced sensitivities between 90% and 100% in the area of 80% to 90% specificity, whereas the receiver operator characteristic curve for CA-125 alone resulted in sensitivities of 70% to 80%. The classification tree analysis for discrimination of benign condition from ovarian cancer used CA-125, granulocyte colony-stimulating factor (G-CSF), IL-6, EGF, and VEGF resulting in 86.5% sensitivity and 93.0% specificity. The presented data show that simultaneous testing of a panel of serum cytokines and CA-125 using LabMAP technology may present a promising approach for ovarian cancer detection. (Cancer Epidemiol Biomarkers Prev 2005;14(4):981–7)

Introduction

Ovarian cancer represents the third most frequent cancer of the female genital tract. The majority of early-stage ovarian cancers are asymptomatic, and over three-quarters of clinical diagnoses are made at a time when the disease has already established regional or distant metastases. Despite aggressive cytoreductive surgery and platinum-based chemotherapy, the 5-year survival for patients with clinically advanced ovarian cancer is only 15% to 20%, in striking contrast to the cure rate for stage I disease, which is usually >90% (1). These statistics provide the primary rationale to improve ovarian cancer screening and early detection.

Due to the low prevalence of spontaneous ovarian cancer in the general population, a screening strategy must have sensitivity of at least 80% in early-stage disease and near-perfect specificity of at least 99.6% (2). At present, there are two screening tests for ovarian cancer: serologic screening for tumor antigen using cancer antigen-125 (CA-125), and imaging using transvaginal sonography (2-6). However, with a cutoff of 30 to 35 units/mL, serum CA-125 has been shown to have a sensitivity of only 50% to 60% with the specificity of >98%, for early-stage disease (4, 7, 8). Transvaginal sonography alone or combined with Doppler and morphologic indices, are only sensitive and specific for established tumors, and are, therefore, not suitable for early diagnostics of ovarian cancer (6, 9). Recently, a novel technology, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry has been offered for early detection of ovarian cancer (10). This technology was reported to allow for discriminating serum protein profiles with 100% sensitivity and 100% specificity (11). However, in two other studies of early detection of ovarian cancer using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, the results were less optimistic, demonstrating 72.8% to 95.7% sensitivity and 82.6% to 94.9% specificity (12, 13). Therefore, at present, proteomic profiling, whereas promising, does not possess the required diagnostic discrimination for primary ovarian cancer screening. Additional approaches are necessary to provide the required high level of specificity and positivity for an effective high-throughput screening for ovarian cancer.

During the last two decades, a large number of serologic tumor markers have been evaluated for their ability to detect early-stage epithelial ovarian cancer. Biomarkers that have a shown association with ovarian cancer include cancer antigens, differentiation markers, antibodies to mutated oncogenes, and cytokines (reviewed in ref. 14). Cytokines are a diverse group of proteins comprised of hematopoietic growth factors, interferons, lymphokines, and chemokines (15). Serum cytokines that possess diagnostic value in ovarian cancer include interleukin (IL)-6, IL-8, macrophage colony-stimulating

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Requests for reprints: Anna E. Lokshin, Hillman Cancer Center, University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-7706; Fax: 412-623-7704. E-mail: lokshina@upmc.edu

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factor (M-CSF), MCP-1, tumor necrosis factor receptor (TNFR), and vascular endothelial growth factor VEGF (refs. 16-21). Cytokines are implicated in many aspects of tumor growth (reviewed in ref. 22). Tumor cells express and produce various angiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), IL-6, IL-8 (23-26), and other cytokines, such as MCP-1, granulocyte CSF (G-CSF), M-CSF, tumor necrosis factor-α (TNFα), IL-1α, and IL-1β (24, 27-29). Tumor-produced cytokines could bind to their receptors expressed by endothelial and hematopoietic/lymphoid cells and trigger production of additional types of cytokines (22). This leads to the accumulation of high concentrations of these factors locally (ascites) as well as systemically (in the blood). Cytokine profiles could be cancer-specific since malignant cells of different histologic types could produce different patterns of proangiogenic factors, growth factors, and chemokines. Thus, cytokine panels could serve as cancer biomarkers that can be used for early diagnosis and assessment of therapy response. Previous publications showed that none of these markers, used alone, is sufficiently diagnostic of malignancy (14, 30). In some studies, combinations of several markers have been evaluated for early detection of ovarian cancer using conventional ELISA assays. Analysis of the diagnostic power of individual serologic markers in combination with CA-125 resulted in increased sensitivity and specificity (3, 14, 30-39). Due to the limitations of ELISA (which is expensive, time-consuming, and each assay encompasses only one marker at a time), none of the tested marker combinations thus far was sufficiently comprehensive and achieved the required characteristics for diagnosis of ovarian cancer. Therefore, further research is necessary to identify a multibiomarker panel allowing for early detection of ovarian cancer with required high sensitivity and specificity.

To test this, we have used a novel multianalyte LabMAP profiling technology (Luminex Corp., Austin, TX), that allows simultaneous measurement of multiple biomarkers in serum or plasma of ovarian cancer patients and control healthy women. In this study, a panel of 24 serologic markers including cytokines, chemokines, growth and angiogenic factors, and CA-125 was analyzed in blood sera of ovarian cancer patients (stages I and II), patients with benign pelvic disease, and control healthy women. Our studies show that a panel of cytokines in combination with CA-125 showed increased specificity and sensitivity as compared with CA-125 alone.

Patients and Methods

Patient Populations. The serum samples from 44 patients diagnosed with early-stage (I and II) ovarian cancer, 37 patients with benign pelvic masses, and 45 healthy age-matched controls were tested. Serum samples from patients with early-stage (I and II) ovarian cancer, and women with benign pelvic disease, were provided by the Gynecologic Oncology Group (Cleveland, OH). Patients were enrolled by the Gynecologic Oncology Group under their Institutional Review Board protocols. Information about gynecologic diagnoses and ovarian cancer staging as well cancer histology and grade was provided by the Gynecologic Oncology Group. No data allowing identification of patients were provided. All major types of epithelial ovarian cancer and a variety of benign pelvic conditions were represented in these series (Table 1). Control serum samples from healthy, age-matched women were received from the Allegheny County Case-Control Network. Written informed consent was obtained from each subject or from his or her guardian. Sample collection was done after approval by the Institutional Review Board and in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services.

Table 1. Patient characteristics

Patient group	Age, median (range)	Histologic types
Control (n = 45)	46 (36-76)	
Early-stage ovarian cancer (n = 44)	46 (34-88)	Papillary serous carcinoma (n = 13) Carcinoma, endometrioid (n = 10) Carcinoma, mucinous (n = 7) Carcinoma, poorly differentiated (n = 6) Adenocarcinoma, serous (n = 5) Carcinoma, clear cell (n = 3)
Benign tumors (n = 37)	44.5 (28-87)	Adenofibroma, serous (n = 1) Brenner tumor (n = 1) Crystadenofibroma, serous (n = 2) Cyst, paratubal (n = 2) Cyst, serous (n = 1) Cyst, simple (n = 3) Cystadenofibroma, serous (n = 3) Cystadenoma, mucinous (n = 8) Cystadenoma, serous (n = 9) Endometriosis (n = 1) Fibrosis (n = 1) Ovary benign (n = 3) Mucinous benign (n = 2)

Collection and Storage of Blood Serum. Ten microliters of peripheral blood was drawn from subjects using standardized phlebotomy procedures. Handling and processing was similar for all three groups of patients. Samples were obtained from patients diagnosed with ovarian cancer, prior to surgery, and before administration of anesthesia. Blood samples were collected without anticoagulant into red top vacutainers and allowed to coagulate for 20 to 30 minutes at room temperature. Sera were separated by centrifugation, and all specimens were immediately aliquoted, frozen and stored in a dedicated −80°C freezer. No more than two freeze-thaw cycles were allowed for each sample.

Multiplex Analysis. The LabMAP technology (Luminex) combines the principle of a sandwich immunoassay with the fluorescent-bead-based technology allowing individual and multiplex analysis of up to 100 different analytes in a single microtiter well (40). The LabMAP serum assays were done in 96-well microplate format according to the protocol by Biosource International (Camarillo, CA). A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 minutes with PBS/bovine serum albumin. To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in serum diluent. Standards and patient sera were pipetted at 50 μL per well in duplicate and mixed with 50 μL of the bead mixture. The microplate was incubated for 1 hour at room temperature on a microtiter shaker. Wells were then washed thrice with washing buffer using a vacuum manifold. Phycoerythrin (PE)-conjugated secondary antibody was added to the appropriate wells and the wells were incubated for 45 minutes in the dark with constant shaking. Wells were washed twice, assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). Analysis of experimental data was done using five-parametric-curve fitting.

Development of LabMAP Assays for CA-125. Assay for CA-125 was developed in our laboratory according to the protocol by Luminex. Antibody pair for CA-125 was purchased from Fitzgerald Industries International

(Concord, MA). Detection antibody were biotinylated using the EZ-Link sulfo-NHS-biotinylation kit (Pierce, Rockford, IL) according to the manufacturer's protocol. The extent of biotin incorporation was determined using the HABA assay (Pierce) and was found to be 20 mol of biotin per mole of protein for all of the biotinylation reactions. The capture antibody was covalently coupled to individual spectrally addressed carboxylated polystyrene microspheres purchased from Luminex. Covalent coupling of the capture antibodies to the microspheres was done by following the procedures recommended by Luminex. In short, microsphere stock solutions were dispersed in a sonication bath (Sonicor Instrument Corporation, Copiaque, NY) for 2 minutes. An aliquot of 2.5 $\times$ 10^6 microspheres was resuspended in microtiter tubes containing 0.1 mol/L sodium phosphate buffer (pH 6.1), to a final volume of 80 μL. This suspension was sonicated until a homogeneous distribution of the microspheres was visually observed. Solutions of *N*-hydroxy-sulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce), both at 50 mg/mL, were prepared in phosphate buffer, and 10 μL of each solution was sequentially added to stabilize the reaction and activate the microspheres. This suspension was incubated for 10 minutes at room temperature and then resuspended in 250 μL of PBS containing 50 μg of antibody. The mixture was incubated at room temperature overnight in the dark with continuous shaking. Microspheres were then incubated with 250 μL of PBS-0.05% Tween 20 for 4 hours. After aspiration, the beads were blocked with 1 mL of PBS-1% bovine serum albumin-0.1% sodium azide. The microspheres were counted with a hemacytometer and stored at a final concentration of 10^6 microspheres/mL in the dark at 4°C. The coupling efficiency of monoclonal antibodies was tested by staining 2,000 microspheres with phycoerythrin-conjugated goat anti-mouse IgG (BD Biosciences, San Diego, CA). Microspheres were analyzed by Bio-Plex system, and the mean fluorescence intensity of >15,000 was accepted as an indicator of sufficient coupling efficiency. The minimum detection levels for CA-125 was <5 international units/mL. Interassay variability, expressed as a coefficient of variation, was calculated based on the average for 10 patient samples and standards that were measured in four separate assays. The interassay variability within the replicates presented as an average coefficient of variation was in the range of 5.4% to 9.1% (data not shown). Intraassay variability was evaluated by testing quadruplicates of each standard and 10 samples measured thrice. The variabilities of these samples were between 5.6% and 9.6% (data not shown). CA-125 assay was further validated in comparison with standard clinical ELISA (Centocor, Malvern, PA) and has shown 94.5% correlation. 24-plex assay for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-18, TNFα, IFNγ, GM-CSF, EGF, VEGF, G-CSF, basic fibroblast growth factor, hepatocyte growth factor (HGF), RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β, and MCP-1 purchased from Biosource International. Interassay variabilities for individual cytokines in 24-plex were in the range of 3.5% to 9.8% and intraassay variabilities were in the range of 3.6% to 12.6% (information provided by Biosource International). Each assay was validated against appropriate ELISA demonstrating 95% to 99% correlations (information provided by Biosource International).

Statistical Analysis of Data. Descriptive statistics and graphical displays (i.e., dot plots) were prepared to show the distribution of each marker for each disease state. The Wilcoxon rank-sum test was used to evaluate the significance of differences in marker expression between each disease state. Spearman's (nonparametric) rank correlation was also calculated to quantify the relationships between each pair of markers.

Discrimination of ovarian cancer status was accomplished using classification and regression trees (CART; ref. 41) implemented through S-Plus statistical software (42). Classification trees discriminate between outcome classes (e.g., cancer patients versus controls) by first searching the range of each potential predictor (e.g., a given cytokine) and finding the split that maximizes the likelihood of the given data set. Within each resulting subset (or node), the algorithm again searches the range of each variable to choose the optimal split. This process is continued until all observations are perfectly discriminated, or the sample size within a given node is too small to divide further (i.e., $n = 5$ or less). Only two observations in the data set had missing values for any of the markers and were excluded from the analysis. The final output of the resulting classification tree is a graphical display of decision criteria for each split and resulting predicted probabilities of being a case across the final splits (i.e., terminal nodes). Several other methods (logistic regression and neural networks) were also implemented with similar, but somewhat less optimal results (results not shown).

Ten-fold cross-validation (43, 44) was implemented to assess classification accuracy using independent data. Specifically, the data were randomly split into 10 subsets of equal size (or as equal as possible; n_k = 8-9 for these data). For each subset, a model was fit to 90% of the data outside that subset; the resulting model (or tree) was then applied to 10% of data within the given subset. The resulting estimate of classification accuracy therefore uses separate subsets of data for model fitting and validation, and thus avoids resubstitution bias. The resulting sensitivity and specificity are reported across a range of decision rules (i.e., cut-points for classifying a given predicted probability as either a case or control) to generate the receiver operator characteristic (ROC) curve. Since cross-validation produces a potentially different model for each subset of the data, however, the classification tree produced using all observations (i.e., without cross-validation) was displayed for purposes of describing the optimal model. When not otherwise stated, observations with a predicted probability >0.5 are classified as a case (or as a benign condition for the comparison of benign versus controls).

Results

LabMAP-Based Analysis of Serum Concentrations of Cytokines and CA-125 in Ovarian Cancer Patients. Concentrations of 24 different serum markers belonging to different functional groups were evaluated in a multiplexed assay using LabMAP technology, in serum samples of patients from three clinical groups: women with early stage (I and II) ovarian cancer, women with benign pelvic masses, and age-matched healthy controls (Table 1). Serum levels of IL-2, IL-4, IL-5, IL-10, IL-13, IL-15, IL-17, IL-18, TNFα, and IFNγ were undetectable in either control or patients' sera. IL-1β, IL-12p40, MIP-1α, MIP-1β, HGF, RANTES, bFGF, and GM-CSF showed measurable serum concentrations which did not differ between the control and patient groups (data not shown). Serum concentrations of IL-6, IL-8, CA-125, and VEGF were found to be significantly higher in ovarian cancer patients as compared with controls ($P < 0.05$ - $P < 0.001$; Table 2; Fig. 1). LabMAP assays showed relatively high serum concentrations of EGF (224 $\pm$ 12 pg/mL) and MCP-1 (384 $\pm$ 21 pg/mL) in the serum of healthy women (Table 2; Fig. 1). However, serum levels of EGF and MCP-1 were significantly lower ($P < 0.05$ - $P < 0.001$) in ovarian cancer patients as compared with controls (Table 2; Fig. 1).

Serum of patients with benign tumors had significantly elevated levels of VEGF ($P < 0.05$), G-CSF ($P < 0.01$), IL-6 ($P < 0.001$), and CA-125 ($P < 0.01$) as compared with controls (Table 2). In addition, patients with benign tumors had

Table 2. Levels of serum markers

Analytes/patients		Healthy controls	Ovarian cancer	Benign
EGF	mean ± SE	223.8 ± 11.46	110.7 ± 15.58***	98.6 ± 12.35***
	median (range)	238 (29.8-402.6)	74.9 (0-396.9)	94.9 (0-276.4)
IL-6	mean ± SE	8.8 ± 2.50	64.2 ± 12.72***	28.0 ± 9.3***
	median (range)	0 (0-64.1)	23.8 (0-280.2)	7.6 (0-275.3)
G-CSF	mean ± SE	21.8 ± 8.44	49.2 ± 12.04^{NS}	77.4 ± 14.04**
	median (range)	0 (0-257.6)	0 (0-290.8)	0 (0-339.1)
IL-8	mean ± SE	10.2 ± 1.68	24.0 ± 5.98**	12.4 ± 3.11
	median (range)	6 (2.3-51.4)	9.6 (2.0-180.6)	7.6 (3.0-127.8)
VEGF	mean ± SE	90.7 ± 10.52	153.5 ± 19.95*	258.8 ± 26.04*
	median (range)	67 (18-306)	106 (28-552)	218 (48-662)
CA-125	mean ± SE	10.4 ± 2.28	153.7 ± 44.04***	51.8 ± 13.23**
	median (range)	6.0 (0-87)	51.0 (0-1412)	16.0 (0-372)
MCP-1	mean ± SE	341.8 ± 21.34	210.3 ± 20.54***	196.3 ± 16.06***
	median (range)	326.8 (135.5-695.7)	172.9 (17.1-502.3)	178.2 (44.9-434.6)

NOTE: Comparison of ovarian cancer or benign patients with controls: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

significantly lower levels of EGF and MCP-1 as compared with controls ($P < 0.001$ for both; Table 2). When cytokine levels were compared between cancer and benign groups, significantly lower circulating concentrations of IL-6, IL-8, and CA-125 were observed in sera of benign cases ($P < 0.05$ for all; Fig. 1). G-CSF concentration was significantly ($P < 0.05$) higher in the benign group as compared with the cancer group (Fig. 1). Patients with benign pelvic disease did not differ from patients with early-stage ovarian cancer with regard to of concentrations of EGF, VEGF, and MCP-1 (Fig. 1).

Statistical Analysis of Serum Cytokines as Ovarian Cancer Biomarkers

Comparison of Early-Stage Ovarian Cancer versus Healthy Controls. Table 3 illustrates classification results using each individual cytokine to distinguish early-stage ovarian cancer from controls. Results show that the individual markers led to only moderately accurate prediction of early-stage cancer. Only CA-125, EGF, and IL-6 correctly classified >80% of the test set subjects (Table 3).

Figure 2A displays the classification tree using CART methodology for discriminating controls from early-stage ovarian cancer. The model in Fig. 2A used all observations in either group to fit the model (as opposed to cross-validation, which is used for subsequent estimation of classification accuracy as explained in subsequent paragraphs). The classification tree used five of the eight markers, including CA-125, EGF, VEGF, IL-6, and IL-8. The numbers specified for each of the final groups (i.e., terminal nodes) represent the probability of being a case within each subset.

Rates of classification accuracy (in discriminating controls from early-stage cancer) were then obtained using 10-fold cross-validation. Figure 2B displays the resulting ROC curve. As described in Patients and Methods, the sensitivity and specificity depend on the cut-point (i.e., predicted probability from the classification tree) used to classify each subject as either a case or control. Using the standard cut-point of 0.5 (i.e., everyone with a predicted probability >0.5 is classified as a cancer case) gives 100% sensitivity, 86% specificity, and 93% correctly classified. Fixing the specificity at 91% still leads to a very high sensitivity, at 96% (again with 93% correctly classified). Alternatively, a specificity of 95.3% corresponds to a sensitivity of 84.1% (and 90.0% correctly classified). The total area under the ROC curve was near one, at 0.966. Additionally, the ROC curve was created using only CA-125 (again based on 10-fold cross-validation; Fig. 2C). Comparing this curve to the combination of markers clearly shows a substantial gain from using multiple markers to predict cancer status. Specifically, in the area of 80% to 90% specificity (i.e., between 0.1 and 0.2 on the x-axis), the final model (using multiple markers) produces sensitivities between 90% and 100%, whereas CA-125 only produces sensitivities in the area of 70% to 80%.

Several models provided comparable high sensitivity and specificity for early diagnosis of ovarian cancer. Therefore, the resulting combination of cytokines should not be viewed as a

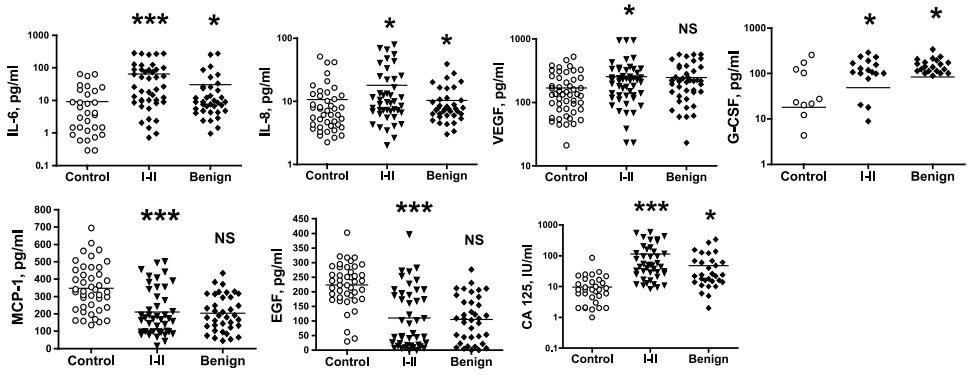

Figure 1. Distribution of serum levels of markers in the three study groups. Serum levels of cytokines and growth factors in healthy controls, ovarian cancer patients at stages I and II and patients with benign gynecologic disease. Sera were collected from 45 patients with early-stage (I and II) ovarian cancer, 44 patients with benign pelvic masses, and from 37 age- and sex-matched healthy controls. Circulating concentrations of cytokines and growth factors were measured using LabMAP technology as described in Patients and Methods. Measurements were done twice. Horizontal lines, mean values; *, statistical significance between controls and cancer patients or between patients with benign pelvic disease and patients with ovarian cancer; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3. Predictive values for single serum markers for early-stage ovarian cancer

Cytokine	% Correctly classified	Sensitivity	Specificity
CA-125	85.1	95.5	74.4
IL-6	85.1	84.1	86.0
EGF	80.5	84.1	76.7
IL-8	79.3	88.6	69.8
MCP	78.2	84.1	72.1
VEGF	73.6	79.5	67.4
G-CSF	73.6	72.7	74.4

unique subset of markers. Other models with the same number of cytokines (data not shown), often led to very similar results. For instance, all of the tested three-variable models led to very similar classification rates. The large number of possible combinations, and the computational demands of iteratively partitioning the training and test sets, prevented an exhaustive search of all possible models.

Comparison of Controls and Early-Stage Ovarian Cancer versus Benign Conditions. To assess the validity of serum biomarker panel for discrimination of benign pelvic tumors from the other groups, separate classification tree models were fit to predict (*a*) benign conditions versus early-stage cancer, and (*b*) benign conditions versus controls. The same 10-fold cross-validation procedure was used to assess classification accuracy. For the comparison of benign versus cancer, 80.2% of subjects were correctly classified, with a sensitivity of 84.1% and a specificity of 75.7%. The classification tree for comparison of benign versus cancer (data not shown) used five markers (CA-125, G-CSF, IL-6, EGF, and VEGF). For the comparison of benign versus controls, 90.0% of subjects were correctly classified, with a sensitivity of 86.5% and a specificity of 93.0%. The classification tree for comparison of benign versus controls (data not shown) used six of the eight markers, including EGF, VEGF, G-CSF, CA-125, IL-6, and IL-8.

Correlation Between Biomarkers. Analysis of correlations between individual markers using Spearman rank correlation method revealed that most of the markers were relatively uncorrelated (Table 4). Only MCP-1 and EGF ($r = 0.45$) had a correlation >0.4; IL-6 and IL-8 ($r = 0.34$) were the only other markers having a correlation >0.3. The majority of markers had a correlation of 0.10 or less (in absolute value), suggesting that marker combinations may provide complementary classification information.

Discussion

We used LabMAP technology for analyzing 24 cytokines and CA-125 antigen in sera of women with early-stage ovarian cancer in comparison with matched healthy controls and patients with benign pelvic tumors. The sensitivity of the LabMAP assay is comparable to ELISA and RIA (40). In fact, in our experiments, circulating levels of all 25 proteins in healthy women were very similar to those measured by ELISA or RIA and that reported in previously published observations (20, 45-50).

We have identified six circulating proteins that showed an association with ovarian cancer, i.e., EGF, MCP-1, CA-125, VEGF, IL-6, and IL-8. We observed two distinct patterns of changes in the sera of women with ovarian cancer: the concentrations of VEGF, IL-6, IL-8, and CA-125 were higher in patients with ovarian cancer, whereas decreased concentrations of EGF and MCP-1 were found in ovarian cancer as compared with the healthy controls. These elevated levels of VEGF, IL-6, IL-8, and CA-125 in the circulation of ovarian cancer patients have been previously reported from studies using conventional ELISA assays (48, 51-53). Increased levels of cytokines in the blood of cancer patients may be due to secretion by malignant or by normal stromal cells, i.e., immune or endothelial cells.

To the best of our knowledge, this study is the first to observe reduced levels of EGF in patients with ovarian cancer and benign pelvic disease. Lower circulating levels of MCP-1 in ovarian cancer as compared with controls were previously reported by Penson et al. (20). Lower serum concentrations of EGF were observed in patients with differentiated thyroid carcinoma (45) and breast cancer, but not in patients with pancreatic, lung, and head and neck

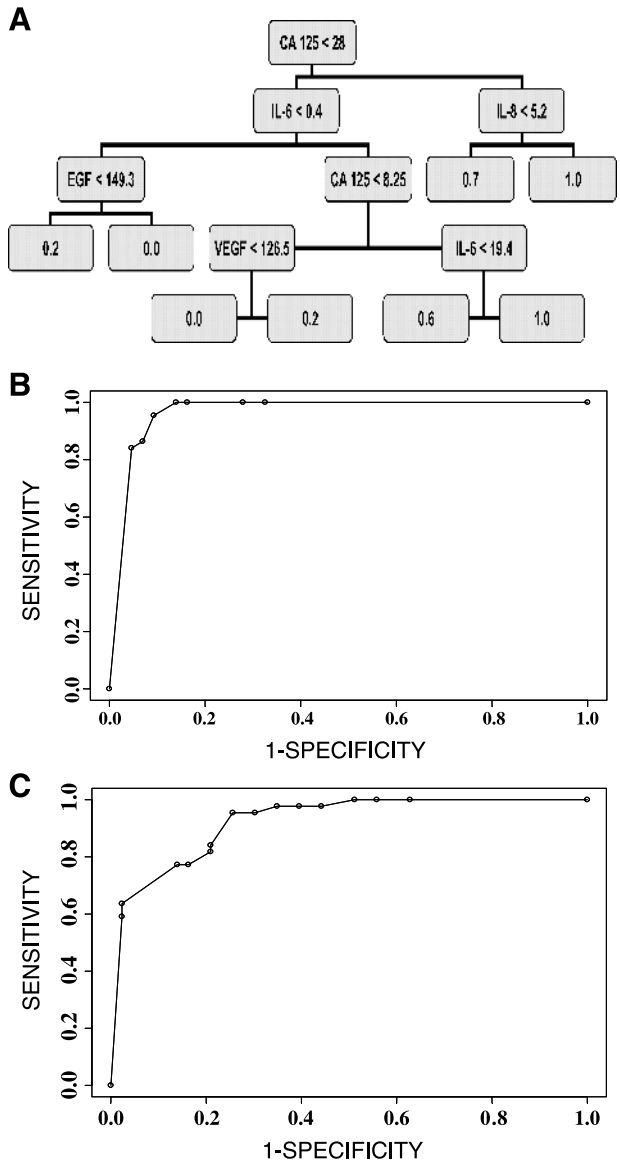

Figure 2. Classification tree and ROC discriminating early-stage ovarian cancer from healthy controls. **A.** Classification tree: rectangles, splitting nodes containing cytokine and cytokine cutoff. The range of data specified at each split represents the subset of data which is further subdivided by branches to the left. The numbers specified for each of the final groups (i.e., terminal nodes) represent the probability of being a case within each subset. **B.** ROC curve for biomarker panel. Results from 10-fold cross-validation of classification tree analysis of early-stage ovarian cancer versus healthy controls. **C.** ROC curve for CA-125 alone. Results from 10-fold cross validation of early-stage ovarian cancer versus healthy controls.

Table 4. Spearman rank correlations

Marker	EGF	V-EGF	MCP-1	G-CSF	CA-125	IL-6	IL-8	IL-12
EGF	1.00							
V-EGF	−0.10	1.00						
MCP-1	0.45	0.01	1.00					
G-CSF	−0.08	0.01	−0.04	1.00				
CA-125	−0.26	0.07	−0.16	−0.05	1.00			
IL-6	−0.07	0.02	−0.06	0.13	0.22	1.00		
IL-8	0.15	0.08	0.21	0.10	0.10	0.34	1.00	
IL-12	0.12	−0.03	0.23	0.13	−0.02	−0.05	0.01	1.00

cancers or melanoma.[6] Therefore, decreased circulating levels of EGF may be specific for particular type(s) of cancer. Ovarian cancer cells express EGF receptor, and EGF is an autocrine growth factor for ovarian cells (54, 55). We have observed the absorption of EGF and MCP-1 by ovarian tumor cells *in vitro* incubated with serum,[6] indicating that lower circulating levels of these molecules in ovarian cancer patients might be due to the consumption by ovarian tumor expressing specific receptors (56, 57).

Analysis of serum biomarkers in patients with benign pelvic masses revealed increased levels of VEGF and G-CSF that could be explained by their proangiogenic effects and stimulation of angiogenesis and formation of a blood vessel network that is essential for supporting growth not only of malignant but also benign tumors. However, in comparison with ovarian cancer serum, no significant increase in IL-8 concentrations in the serum of patients with benign tumors was observed. The serum levels of IL-6 and CA-125 were found to be elevated in patients with benign tumors but not to the same extent as in patients with ovarian cancer. Decreased concentrations of EGF and MCP-1 were found to be similar in the serum of patients with benign masses and ovarian cancer. It is possible that receptors for EGF and MCP-1 are also expressed by benign tumor cells. Expression of EGFR by normal epithelial cells has been reported (55). Discrimination of ovarian cancer from benign samples has presented a difficult problem in past studies, in which a relatively high percentage of false-positive classification of benign neoplasms has been observed (12, 58).

Statistical analysis showed that although correlation of each of the above markers with ovarian cancer was modest when evaluated alone, a combined biomarker panel showed very strong association with malignant disease, and can have potential utility for early diagnosis of ovarian cancer. Combinations of several serum markers as measured by LabMAP technology provided a sensitivity of 84% at a specificity of 95% in this sample set. Due to the low prevalence of spontaneous ovarian cancer in the general population, a screening strategy must have sensitivity of at least 80% in early-stage disease and near-perfect specificity of at least 99.6% (2). We should therefore ideally evaluate the sensitivity of a given model using a cutoff that produces very high specificity. Sensitivities are reported here for fixed specificities as high as 95%. Although reporting the model's sensitivity at higher specificities (e.g., 98% or 99%) would be preferable, such results could not be reached (with the given model and the given data sets) due to the small number of controls. We expect to improve on such results through the future collection of larger data sets and an expanded panel of ovarian-associated biomarkers. It should also be noted that the 100% sensitivity and specificity results apply to samples obtained from ovarian cancer cases already clinically diagnosed, along with healthy controls, and not from a prospective screening trial. The results in a prospective screening trial are likely to be lower than obtained with preoperative samples. These results, however, show a strong potential to accurately discriminate cancer status with only a moderate number of samples.

For an estimate of the optimal classification tree, we presented the model fit to the entire data set, which is subsequently referred to as the overall model. It should be noted that the cross-validation procedure used here produces a different model for each of the 10 training data sets. Each of these 10 classification trees, however, was either the same as, or subsets of the overall model. None of the 10 models fit through the cross-validation procedure included any markers that were not in the overall model (i.e., MCP-1 or G-CSF). Seven of the 10 cross-validation models included four of the five markers in the overall model. Although some bias may result from this cross-validation procedure, as opposed to separate training and test sets, the latter approach is not feasible unless one has very large sample sizes. With the given sample sizes, separate training and test sets would lead to more unstable estimates of sensitivity and specificity, since each observation can only be used for training or prediction. For the given data, the 10-fold cross-validation approach represents a reasonable alternative to at least partially avoid classification bias (imposed when the same data are used from both training and prediction), and estimate classification measures (e.g., sensitivity and specificity) with improved precision. However, whereas this approach substantially reduces the problem of classification bias, it does not easily allow ready calculation of confidence limits, which is a limitation of the cross-classification approach, compared with splitting the data into two independent training and test sets.

The predictive power of combined serologic markers for early-stage ovarian cancer, as determined by LabMAP technology, is at least as good as that identified in published studies for surface-enhanced laser desorption/ionization time-of-flight mass spectrometry technology (12). However, LabMAP technology is less expensive and permits a high-throughput approach. Furthermore, the flexibility of LabMAP technology allows for the addition of new markers and therefore for the opportunity to incrementally increase the diagnostic power of the combined assay. To the best of our knowledge, the reported multiplexed cytokines/CA-125 offers the highest predictive power, as compared with other published assays using defined protein serologic markers (3, 14, 30, 32-39, 59-62).

In conclusion, we show here that analysis of multiple serum biomarkers using a novel LabMAP technology is a promising approach for the development of a diagnostic assay for ovarian cancer. The predictive power of the cytokines/CA-125 panel is still lower than that required for general population ovarian cancer screening as defined by Jacobs (2). Optimization of cytokine/CA-125 panel by including additional markers with high association with ovarian cancer would likely increase the diagnostic power of the assay. Further validation of this assay in retrospective studies with a larger number of samples will allow for confirming the clinical diagnostic utility of LabMAP-based assay.

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Circulating IL-8 and anti-IL-8 autoantibody in patients with ovarian cancer

Anna E. Lokshin [a,b,*], Mathew Winans [a], Douglas Landsittel [a], Adele M. Marrangoni [a], Lyudmila Velikokhatnaya [a], Francesmary Modugno [a,c], Brian M. Nolen [a], Elieser Gorelik [a,d]

[a] *University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[b] *Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[c] *Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[d] *Department of Pathology and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA*

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Abstract

Objectives. In an ongoing effort to identify diagnostic ovarian cancer biomarkers, SEREX (serological analysis of recombinant cDNA expression libraries) technique was employed resulting in detection of 20 known genes, nine ESTs and one novel sequence. Interleukin-8 (IL-8) was one of ovarian cancer-associated antigens identified by SEREX screening. The objective of this study was, therefore, to evaluate the potential importance of circulating anti-IL-8 antibody as ovarian cancer biomarker.

Methods. We developed and optimized a new immunofluorescent bead-based assay for detection of anti-IL-8 antibody in blood serum. Circulating IL-8 and anti-IL-8 IgG concentrations were measured in blood sera from 44 patients with early stage (I–II) ovarian cancer, 50 patients with late stage (III–IV) ovarian cancer, 37 patients with benign pelvic masses, and 80 healthy women using the bead-based assay.

Results. Our data indicate that serum contains IL-8 cytokine, anti-IL-8 antibody, and IL-8:anti-IL-8 complexes. We found that concentrations of IL-8 and anti-IL-8 antibody were elevated in sera of patients with ovarian cancer as compared with healthy controls. Logistic regression analysis of circulating concentrations of anti-IL-8 IgG in patients with stages I–II ovarian cancer versus healthy controls allowed for prediction of early ovarian cancer with 98% specificity, 65.5% sensitivity, 80.3% of patients correctly classified. Combining IL-8 and anti-IL-8 IgG with CA 125 resulted in increased classification power as compared to individual markers analyzed separately.

Conclusion. Thus, IL-8 and anti-IL-8 autoantibody might potentially serve as additional biomarkers for ovarian cancer.
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Keywords: Ovarian cancer; IL-8 autoantibody; Diagnostic

Introduction

Increasing evidence indicates that malignant tumors could induce T-cell-mediated and humoral responses. In the last decade, various T cell recognizable tumor-associated antigens have been identified [1]. The ability of cancer patients to generate antibodies to various oncogene products has been also well documented [2–7]. Progress in the identification of antitumor antibodies and their potential targets was substantially accelerated by the development of the SEREX technique, which is based on cloning a tumor cDNA library into λZAP bacteriophage and identifying nucleotide sequences and gene products reacting with cancer patients' serum [8–11]. With the use of SEREX, antibodies to several antigens such as the lysosomal protease cathepsin D, NY-ESO-1 and MAGE-1, homeobox protein HOXB6 and 7, TOP2A, and others have been reported in ovarian cancer patients [9,12]. In the present study, we used SEREX and cDNA of ovarian cancer to further search for cancer antigens in sera of ovarian cancer patients. We have identified additional 14 antigens in these sera, including IL-8, dolichyl phosphate glucosyltransferase, coenzyme A oxidase, ribosomal protein L14 (RPL14), and MO25 protein.

IL-8 belongs to the superfamily of CXC chemokines attracting neutrophils and macrophages and manifests a wide range of proinflammatory effects [13]. In addition, IL-8 was

* Corresponding author. University of Pittsburgh Cancer Institute, Hillman Cancer Center, 5117 Centre Ave, Pittsburgh, PA 15213, USA. Fax: +1 412 623 7704.
E-mail address: lokshina@pitt.edu (A.E. Lokshin).

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found to be a potent proangiogenic factor and is able to promote tumor cell proliferation, modulate collagenase production, and affect metastasis formation [14]. IL-8 is produced by various malignant cells, and increased blood levels of IL-8 have been demonstrated in several malignancies including ovarian cancer [15–20]. Our SEREX analysis indicates that serum of ovarian cancer patients contains anti-IL-8 antibody. Although an increased concentration of IL-8 in ovarian cancer patients has been previously demonstrated, production of anti-IL-8 antibody in ovarian cancer patients has not been reported. In the present study, we report the development of a new immunofluorescent bead-based assay for detection and evaluation of diagnostic power of autoanti-IL-8 antibody in the serum of ovarian cancer patients.

Materials and methods

Human serum samples

For SEREX screening, serum samples from 20 patients with stages III–IV ovarian cancer were obtained at Magee-Womens Hospital of Pittsburgh (IRB protocol #MWH-98-073) during routine diagnostic or therapeutic procedures. In addition, serum samples from 44 patients diagnosed with early (I–II) stage ovarian cancer, 50 patients diagnosed with late (III–IV) stage ovarian cancer, and 37 patients with benign pelvic masses, and 35 healthy controls were provided by the Gynecologic Oncology Group (GOG) (Cleveland, OH) without individual identification of patients. Control serum samples from 45 healthy, age-matched women were received from the Allegheny County Case-Control Network. Written informed consent was obtained from each subject.

Collection and storage of blood specimens

Ten milliliters of peripheral blood was drawn from subjects using standardized phlebotomy procedures. Blood samples were collected without anticoagulant into two 5 ml red top vacutainers. After blood coagulation, sera were separated by centrifugation, and all specimens were immediately frozen and stored in a dedicated −80°C freezer. All blood samples were logged into the study computer to track information such as storage date, freeze/thaw cycles, and distribution. Blood samples from patients with ovarian cancer were collected prior to surgery, anesthesia, or chemotherapy.

Immunoscreening of the ovarian cancer cDNA library

An ovarian cancer cDNA library cloned into the λZAP bacteriophage expression vector was purchased from Stratagene Corp. (LaJolla, CA). The library was derived from a papillary serous carcinoma isolated from ascites. *E. coli* (XL1-Blue MRF' strain) transfected with recombinant λZAP phages were plated onto 625-cm^2 NZY-agar plates. Expression of recombinant proteins was induced with isopropyl β-D-thiogalactoside (IPTG). Plates containing the phage plaques at a density of 70,000–80,000 per plate were incubated at 41°C until plaques were visible and then blotted onto nitrocellulose membranes for 4 h at 41°C. Serum samples from 20 patients from Magee-Womens Hospital with ovarian cancer were pooled, preabsorbed with transfected *E. coli* lysate, and further diluted to 1:2000. Membranes spotted with SEREX ovarian cancer antigens were screened with serum IgG as follows. Membranes were blocked for 1 h with 5% dry milk at room temperature followed by overnight incubation with diluted IgG at 4°C. After being washed, the membranes were incubated with horseradish peroxidase-conjugated goat anti-human Fc secondary antibody, and the reactive phage plaques were visualized using an enhanced chemiluminescence (ECL) technique. Membranes were re-screened with pooled, preabsorbed sera from 20 healthy controls to exclude clones that were not specific for ovarian cancer and also the clones that represent IgG that are non-specifically recognized by secondary antibody. Positive clones were cored from the agar plate, and the phage clones were allowed to grow overnight. *E. coli* was infected with isolated phage clones and re-plated in semi-solid agar to ensure monoclonality. Positive phage clones were isolated as above, grown overnight, and followed by a mass excision procedure (Stratagene) to isolate plasmid forms. Plasmid DNA was prepared using Spin-Mini-Prep (Qiagen, Valencia, CA), and inserted DNA was evaluated by *TacI* restriction mapping to identify clones with non-repetitive sequences.

Nucleotide sequence analysis

The nucleotide sequences of isolated cDNA inserts were determined by fluorescence sequencing using an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA), and the resulting gel images were analyzed using the GENESCAN software package. Sequence alignments were performed with BLAST software on GenBank databases.

Coupling of proteins to Luminex microspheres

Purified human recombinant (hr) IL-8 (PeproTech, Inc, Rocky Hill, NJ) and rabbit antibodies raised against human IgG or IgM were covalently coupled to individually spectrally addressed microspheres purchased from Luminex Corporation (Austin, TX) as previously described [21].

Development and optimization of immunofluorescent bead-based assay for detection of anti-IL-8 IgG and IgM

Assays were performed in filter-bottom 96-well microplates (Millipore). IL-8-coupled beads were preincubated with blocking buffer containing 4% bovine serum albumin (BSA) for 1 h at room temperature on microtiter shaker. Beads were then washed three times with washing buffer (PBS, 1% BSA, 0.05% Tween 20) using a vacuum manifold followed by incubation of washed beads with 50 μl blood serum diluted 1:250 for 30 min at 4°C. This dilution was selected as optimal for recovery of anti-IL-8 IgG or IgM based on previous serum titration (data not shown). The washing procedure was repeated, and beads were incubated with 50 μl/well of 4 μg/ml PE-conjugated donkey antibody raised against human IgG or human IgM (Jackson Laboratories), for 45 min in the dark with constant shaking.

For standard curves, Luminex beads were coupled to rabbit antibody generated against human IgG or rabbit antibody generated against human IgM (Jackson Laboratories). Standards representing serial dilutions of human IgG or IgM prepared in serum diluent were mixed at 50 μl/well with 50 μl of beads coupled to rabbit anti-human IgG or IgM and incubated for 30 min at 4°C. Plates were washed using a vacuum manifold, followed by incubation with 4 μg/ml PE-conjugated donkey antibodies raised against human IgG or IgM. Wells were washed twice, assay buffer was added to each well, and bead-associated fluorescence was measured using a Bio-Plex reader (Bio-Rad, Hercules, CA). Data analysis was performed using five-parameter-curve fitting. Assay sensitivity was determined as the lowest detection point. To determine assay specificity, hrIL-8-coupled Luminex beads were incubated with unrelated anti-IL-6, anti-IL-10, or anti-IL-12p40 antibodies, followed by 4 μg/ml of PE-conjugated rabbit anti-human IgG.

To further ascertain assay specificity, absorption assays were performed as follows. hrIL-8 and hr-IL-6 were covalently coupled to carboxylate-modified latex/polystyrene beads (Sigma Chemical Co.) as described above for Luminex beads. Pooled serum from ovarian cancer patients was diluted 1:250, and 50 μl was preincubated with IL-8 or IL-6-coupled Sigma beads for 1 h at 4°C. Additionally, another 50 μl of 1:250 diluted sera was preincubated with Protein A/G Sepharose (Calbiochem) to absorb all IgG. Beads were then removed by centrifugation, and preabsorbed sera were analyzed for the presence of anti-IL-8 IgG using Luminex beads coupled with hrIL-8 as described above.

LabMAP assay for IL-8 and CA 125

An IL-8 assay kit was purchased from BioSource International (Camarillo, CA), and the assay was performed according to the manufacturer's protocol. The CA 125-coupled microbeads were developed in our laboratory, and CA 125 detection assay was performed as described earlier [21]. Analysis of experimental data was performed using five-parameter-curve fitting.

Detection of anti-IL-8:IL-8 complexes

Luminex beads were conjugated with mouse anti-human IL-8 IgG (Biosource International). Beads were incubated with blood sera diluted 1:2 in serum diluent for 1 h at room temperature. Beads were washed and incubated with PE-conjugated donkey IgG raised against human IgG that were additionally preabsorbed with mouse IgG to eliminate any possibility of cross-reactivity. Beads conjugated with irrelevant mouse IgG were used as control.

Sera of ovarian cancer patients with high and low concentrations of IL-8 were absorbed with Protein A/G agarose to remove free IgG as well as IgG complexed with IL-8. Serum (100 μl) was incubated with 30 μl of Protein A/G agarose (Pierce, Rockford, IL) for 30 min at room temperature. After centrifugation (5 min at 800 *g*), serum was removed, and concentrations of IL-8 in the preabsorbed serum were determined using LabMAP assay. To evaluate the ability of IL-8 to form complexes with anti-IL-8 antibody, sera of two healthy women that contain anti-IL-8 antibody, but not IL-8, were spiked with 8 ng/ml of IL-8. To estimate the proportion of the formed complexes in these sera, a LabMAP assay was performed using the pair of anti-IL-8 antibodies that do not recognize antibody-bound IL-8 (PeproTech, Inc., Rocky Hill, NJ).

Statistical analysis

Descriptive statistics and graphical displays (i.e., dot plots) were prepared to show the distribution of each marker for each disease state. Establishing statistical significance of a single marker was performed by the Wilcoxon rank-sum test, also referred to as the Mann–Whitney test. The level of significance was taken as $P < 0.05$. To evaluate the diagnostic value of IL-8 and anti-IL-8 antibody in comparison with CA 125, the data were first randomly split into a training set and a test set of approximately equal size. For each comparison of interest, a logistic regression model [22] was first fit to the training data; predicted probabilities and classification results were then obtained using the independent test set. The random selection of test and training data was repeated 1000 times for each model to obtain valid estimates for the variability of classification rates. Results were described in terms of the mean (across all 1000 random partitions of the training and test sets) percent correctly classified (PCC), sensitivity (SEN), and specificity (SPC). The 95% confidence intervals (95% CI) for PCC, SEN, and SPC were also displayed as the 2.5 and 97.5 percentiles of the distribution. All statistical analyses were conducted using S-Plus statistical software (Seattle, Washington: Math Soft, Inc., 1999). Using coefficients from the logistic model, as fit to the training data, the predicted probability of being a case was then calculated for each subject in the test set. When comparing the ROC curves of two markers, statistical significance was evaluated using a two-sample variant of the Wilcoxon rank-sum test [23].

Results

Identification of ovarian tumor antigens and circulating antibodies by SEREX immunoscreening

To identify ovarian tumor antigens and circulating antibodies that might be useful for detection of ovarian cancer, SEREX immunoscreening was conducted using pooled sera from 20 patients with stages III–IV ovarian cancer (mixed histologies). A commercially available ovarian cancer DNA library representing papillary serous carcinoma isolated from ascites was utilized for this study. Pooled sera were used to identify only shared ovarian cancer-associated antigens while diluting ones that were specific to individual patients. The screening procedure was performed several times, resulting in screening of 8×10^5 phage plaques. Membranes were stripped and re-probed with pooled normal sera and secondary antibody alone to identify and exclude false-positives and cancer-unrelated plaques. True-positive immunoreactive clones were classified as candidate ovarian tumor antigens, sequenced, and the sequence alignments were performed using BLAST software. In total, excluding the false positive clones encoding IgG fragments, primary SEREX screening of the ovarian cancer cDNA library resulted in identification 20 known genes, nine ESTs and one novel sequence (Table 1). Of these, six sequences, i.e., p58 protein kinase, CDC42 binding protein, nuclease sensitive element binding protein 1, ribosomal protein S2 (RPS2), ribosomal protein S4 (RPS4), and a novel sequence, were registered previously in a SEREX database (http://www.licr.org/SEREX.html). Fourteen other sequences have not been previously identified by SEREX as cancer antigens, including IL-8, dolichyl phosphate glucosyltransferase, coenzyme A oxidase, ribosomal protein L14 (RPL14), and MO25 protein (Table 1). IL-8 is a chemokine that is also known for its high proangiogenic activity, and it is highly expressed by ovarian cancer cells [18,20]. Our SEREX data indicate that IL-8 could be a potential target for the host immune response, resulting in the generation of the anti-IL-8 antibody. Therefore, our further experiments were focused on the investigation of IL-8 and anti-IL-8 antibody in sera of ovarian cancer patients.

Development and optimization of immunofluorescent bead-based assay for detection of anti-IL-8 IgG and IgM

To evaluate anti-IL-8 antibody in the serum of ovarian cancer patients, hrIL-8 was coupled to the surface of Luminex beads as described in Materials and methods. Bead-bound hrIL-8 absorbs

Table 1
Analysis of ovarian cancer-associated antigens identified by SEREX

	Gene/Accession #	SEREX database
1	IL-8	–
2	P58 protein kinase	Testis
3	CDC42 binding protein	Breast, ovarian
4	Dolichyl phosphate glucosyltransferase	–
5	Coenzyme A oxidase	–
6	Nuclease sensitive element binding protein 1	Testis
7	MO25 protein	–
8	Ribosomal protein S2 (RPS2)	Ovarian, melanoma, breast, testis, etc.
9	Ribosomal protein L14 (RPL14)	–
10	Ribosomal protein S4 (RPS4)	Ovarian
11	Hypothetical protein FLJ20080	–
12	Hypothetical protein FLJ13346	–
13	Hypothetical protein BC000606	–
14	HNC63-1-G3.R human normal cartilage	–
15	QV1-UT0094-121000-419-b06 EST	–
16	NIH_MCG_77 cDNA, 602575526F1, EST	–
17	Soares infant brain 1NIB, R599767, EST	–
18	NIH_MCG_77 cDNA, BG547680, EST	–
19	NIH_MCG_21 cDNA, 602526754F1, EST	–
20	Unknown	Prostate, melanoma, ovarian, testis, etc.

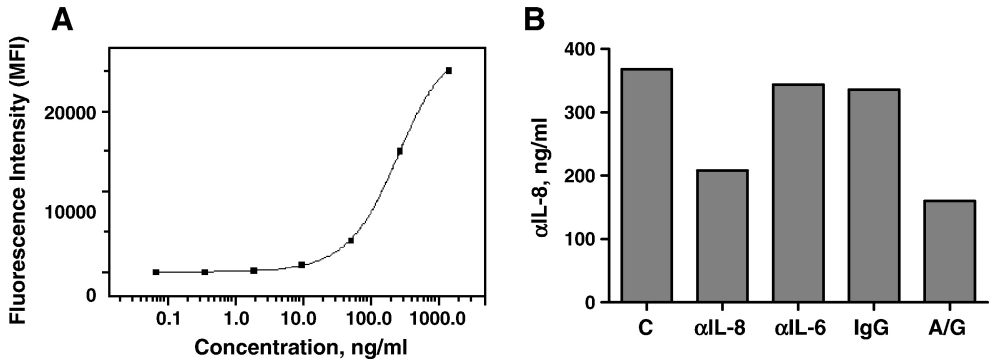

Fig. 1. Development of immunobead-based assay for anti-IL-8 antibody. (A) Standard curve demonstrating the sensitivity of anti-IL-8 antibody assay; (B) competition assay. Pooled 1:250 diluted serum was preincubated with rhIL-8 or rhIL-6-coupled Sigma beads or with Protein A/G Sepharose (A/G). Preabsorbed sera were analyzed for presence of anti-IL-8 IgG.

circulating anti-IL-8 autoantibodies that can be visualized by incubating the beads with PE-conjugated rabbit anti-human IgG or IgM. Bead-associated fluorescence proportional to the concentrations of anti-IL-8 IgG and IgM in tested serum is then measured with a Bio-Plex reader. A LabMAP assay for anti-IL-8 IgG demonstrated sensitivity at least 1 ng/ml (Fig. 1A). To determine assay specificity, hrIL-8-coupled Luminex beads were incubated with unrelated anti-IL-6, anti-IL-10, and anti-IL-12p40 antibodies, followed by 4 μg/ml of PE-conjugated rabbit anti-human IgG. Fluorescence detected with each of unrelated Abs did not exceed the background level (data not shown) confirming that hrIL-8 coupled beads specifically bind anti-IL-8 antibody.

To further ascertain assay specificity, absorption assays were performed as described in Materials and methods. Pooled serum of ovarian cancer patients was preincubated with hrIL-8 or hrIL-6 coupled to polystyrene beads or with Protein A/G Sepharose beads. Beads were then removed by centrifugation, and preabsorbed sera were analyzed for the presence of anti-IL-8 IgG using Luminex beads coupled with hrIL-8 as described above. As shown in Fig. 1B, hrIL-8- and protein A/G-coupled beads, but not hrIL-6-coupled beads, efficiently removed anti-IL-8 IgG from patient serum. The above experiments confirm that immunofluorescent bead-based assays for anti-IL-8 IgG are both sensitive and specific and can be used for direct measurement of circulating anti-IL-8 IgG in blood serum.

Screening for circulating anti-IL-8 antibodies using LUMINEX technology

The newly developed immunofluorescent bead-based assay for anti-IL-8 antibody was then used for screening serum samples obtained from 44 patients with early stage (I–II) ovarian cancer, 50 patients with late stage (III–IV) ovarian cancer, 37 patients with benign pelvic masses, and 80 healthy women. The concentrations of anti-IL-8 IgG and IgM have been determined. The results are presented in Table 2 and Fig. 2. Concentrations of anti-IL-8 IgG in ovarian cancer sera varied from 2.8 to 250.7 ng/ml. Mean concentrations of anti-IL-8 IgG were significantly higher in patients with both early ($P < 0.01$) and late ($P < 0.05$) ovarian cancer as compared with healthy controls (Fig. 2A, Table 2) but were also elevated in the sera of patients with benign pelvic masses. When concentrations of anti-IL-8 autoantibodies were analyzed in a group of patients with only stage I ovarian cancer, the differences between cancer and control groups did not reach statistically significant levels (data not shown).

All three patient groups showed significantly higher ($P < 0.05$–$P < 0.001$) concentrations of IL-8 and CA 125 as compared to healthy controls (Figs. 2B–C). We have additionally performed comparison of concentrations of anti-IL-8 IgG, IL-8, and CA 125 between the two control groups, 35 samples provided by the GOG Blood Bank, and 45 samples obtained from the Allegheny Case Control Network. This study demonstrated identical distributions of anti-IL-8 IgG, IL-8, and CA 125 (data not shown) indicating the comparability of samples from GOG Blood Bank and Allegheny Case Control Network.

Anti-IL-8 IgG concentrations did not differ significantly between patients with early (I–II) versus late stages (II–IV) of ovarian cancer. In comparison to IgG levels of circulating anti-IL-8, IgM was found in to be lower in all tested sera (Table 2). This might be due to the isotype switch and differentiation of IgM producing into IgG producing B cells. Calculations of the

Table 2
Circulating concentrations of anti IL-8 IgG and IgM

	Control		I–II		III–IV		Benign	
	IgG, ng/ml	IgM, ng/ml	IgG, ng/ml	IgM, ng/ml	IgG, ng/ml	IgM, ng/ml	IgG, ng/ml	IgM, ng/ml
Mean	25.4 ± 13.32	12.0 ± 8.73	32.5 ± 13.64	9.2 ± 5.85	35.8 ± 13.21	7.7 ± 4.10	47.0 ± 17.06	18.0 ± 12.01
Min	5.0	1.1	2.8	1.2	2.7	0.9	3.5	2.8
Max	260.0	47.1	250.7	38.5	187.4	32.6	267.3	66.8
IgG:IgM ratio		2.1		3.5		4.6		2.6

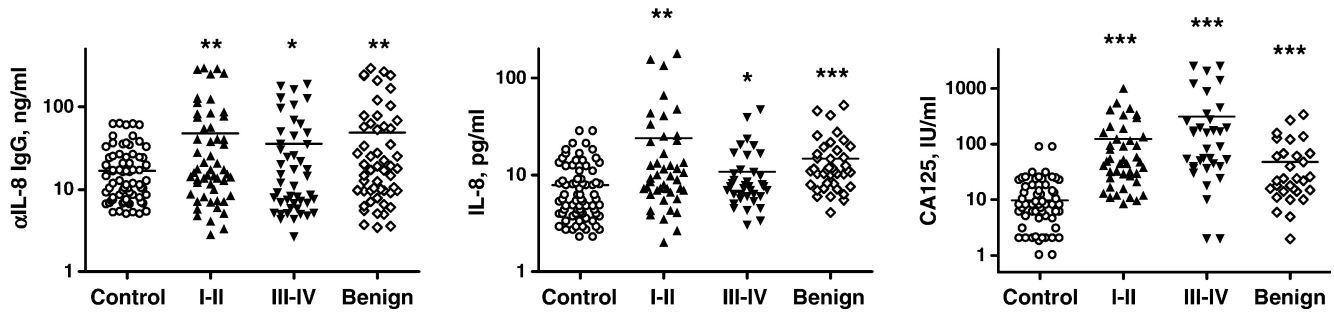

Fig. 2. Serum levels of anti-IL-8 IgG, IL-8, and CA 125 in healthy controls, ovarian cancer patients, and patients with benign pelvic masses. Sera were collected from 44 patients with early stages (I–II) ovarian cancer, 50 patients with late stages (III–IV) ovarian cancer, 37 patients with benign pelvic masses and from 80 age-matched healthy women. Circulating concentrations of autoantibodies, IL-8, and CA 125 were measured using LabMAP technology as described in Materials and methods. Measurements were performed twice. Horizontal lines indicate mean values. *Denotes statistical significance between controls and cancer patients of $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

IgG:IgM ratio revealed that the IgG:IgM ratio in ovarian patients with early and late stages was found to be higher (3.5 and 4.6, respectively) in comparison to 2.1 and 2.6 in sera of control healthy women and women with benign pelvic mass, respectively. These results might be indicative of a more active anti-IL-8 IgG production process in ovarian cancer patients versus control healthy women.

Analysis of IL-8/anti-IL-8 complexes

Simultaneous detection of circulating IL-8 and anti-IL-8 antibody suggest the formation of IL-8-anti-IL-8 complexes. To evaluate this possibility, the following two experiments were conducted. In the first, sera of two ovarian cancer patients with high and low concentrations of IL-8 were absorbed with Protein A/G agarose to remove free IgG as well as IgG complexed with IL-8. Absorption of immunoglobulins by Protein A/G resulted in a 30% decrease in measurable IL-8 concentrations (Fig. 3A). These data indicate that in serum of these two ovarian cancer patients approximately 30% of circulating IL-8 was bound to anti-IL-8 IgG.

In the next experiment, 8 ng/ml of IL-8 was added to the sera of two healthy women that contained measurable anti-IL-8 IgG

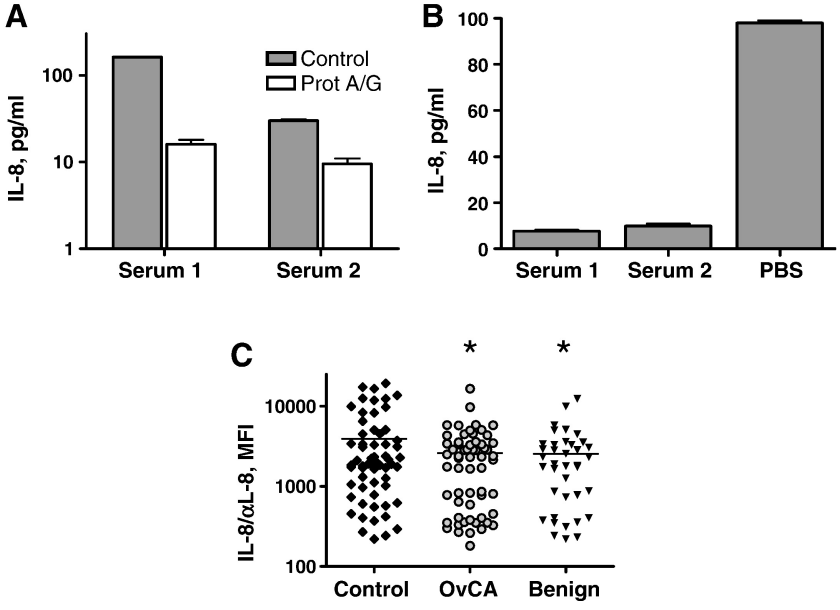

Fig. 3. Analysis of IL-8/anti-IL-8 complexes in serum. (A) Preabsorption with Protein A/G efficiently removes IL-8 complexed with IgG from serum. Sera from two ovarian cancer patients were incubated with Protein A/G agarose to deplete IgG and IgG-IL-8 complexes. IL-8 concentrations were measured before and after incubation. (B) In vitro formation of IgG-IL-8 complexes in serum. HrIL-8 was added at 8 ng/ml to PBS or sera from two healthy donors that contained anti-IL-8 Ab but not IL-8. Formation of IL-8-antiIL-8 complexes was determined by testing IL-8 concentrations using a LabMAP assay with an anti-IL-8 antibody pair that does not recognize antibody-bound IL-8. (C) Analysis of IL-8/anti-IL-8 complexes in serum of patients with ovarian cancer, benign pelvic disease, and healthy women. Complexes were analyzed in blood sera of 50 patients with stages III–IV ovarian cancer, 37 women with benign pelvic disease, and 80 healthy women. Luminex microbeads coupled with mouse monoclonal anti-IL-8 antibody were incubated with 1:2 diluted patients' sera. After washing step beads were further incubated with PE-conjugated donkey anti-human anti-IgG antibody. Data are presented as mean fluorescent intensity (MFI). Horizontal lines indicate mean values. *Denotes statistical significance between controls and patients of $P < 0.05$.

without any detectable IL-8. To estimate the proportion of the formed complexes, a LabMAP assay was performed using the pair of anti-IL-8 antibodies that do not recognize antibody-bound IL-8 (PeproTech, Inc., Rocky Hill, NJ). These anti-IL-8 antibodies detected only about 10% of 'spiked' IL-8, indicating that serum anti-IL-8 antibody is capable of binding IL-8 and formed complexes. In contrast, in PBS 100% of added IL-8 was detected (Fig. 3B).

Next, concentrations of immune complexes were analyzed in serum samples from 50 patients with stages III–IV ovarian cancer, 37 patients with benign pelvic disease, and 80 healthy women. The titers of immune complexes were slightly lower ($P < 0.05$) that those in control groups (Fig. 3C). No differences were observed between concentrations of complexes in patients with ovarian cancer and benign pelvic disease (Fig. 3C). Thus, our data indicate that serum of healthy women and ovarian cancer patients could contain IL-8 and anti-IL-8 antibody and form IL-8/anti-IL-8 complexes.

Predictive values for IL-8, anti-IL-8, and CA 125 for early stage ovarian cancer

Increased serum concentrations of anti-IL-8 antibody in serum of ovarian cancer patients suggest its potential usefulness as a biomarker of ovarian cancer. Logistic regression analysis of circulating concentrations of anti-IL-8 IgG in patients with stages I–II ovarian cancer versus healthy controls allowed for prediction of early ovarian cancer with 98% specificity, 65.5% sensitivity, 80.3% of patients correctly classified (Table 3). The total area under the receiver operating characteristic (ROC) curve was at 0.867 (Fig. 4). In comparison, predictive value of IL-8 showed at 98% specificity, 62.6% sensitivity, and 79.3% of patients correctly classified with the total area under the receiver operating characteristic (ROC) curve being 0.812. The predictive value of CA 125 was at 98% specificity, 76.8% sensitivity, and 85.1% correctly classified subjects (Table 3), and the total area under the receiver operating characteristic (ROC) curve was at 0.921. Concentrations of anti-IL-8 IgG did not correlate with either IL-8 or CA 125 in healthy women and in the three patient groups ($r < 0.2$, data not shown). Thus, it appears that serum IL-8 autoantibody and levels of IL-8 and CA 125 may represent complementary markers. In fact, the combination of IL-8, anti-IL-8 IgG, and CA 125 resulted in increased classification power as compared to any of the markers analyzed separately with the sensitivity of 87.5% and 91.2% correctly classified women at 98% specificity (Table 3). The AUC differences between CA 125 and composite 3-marker curves were significant ($P < 0.05$) for comparison between cancer and control groups. Comparison of patients with stages I–II ovarian cancer to patients with benign pelvic masses using the 3-marker composite panel resulted in 98% specificity, 42% sensitivity, 58% correctly classified. Therefore, the diagnostic power of 3-marker panel for discrimination of benign versus cancer conditions although slightly higher that that of each individual biomarker (data not shown) was very low.

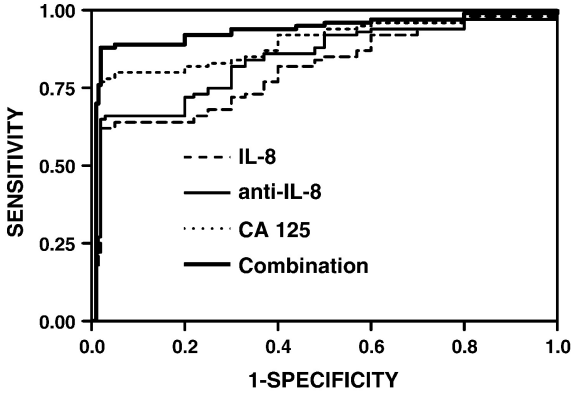

Fig. 4. Receiver operating characteristic (ROC) curves discriminating ovarian cancer from healthy controls. ROC curves are presented for biomarker IL-8, anti-IL-8, CA 125, and the combination of three biomarkers.

Discussion

In the present study, we demonstrate that SEREX immunoscreening identified IL-8 as an ovarian cancer-associated autoantigen. IL-8 demonstrates a strong link with ovarian cancer. It is overexpressed and produced by malignant ovarian tumors [18,24]. IL-8 could stimulate tumor angiogenesis and directly stimulate proliferation of human ovarian cancer cells, indicating a function for IL-8 in the biology of epithelial ovarian cancer [15]. The increased production of IL-8 in ovarian cancer patients leads to increased generation of anti-IL-8 antibodies. We report here the presence of both anti-IL-8 IgG and IgM in blood serum of healthy women and patients with early and late stages of ovarian cancer. The total concentrations of anti-IL-8 IgG and the proportion of IgG:IgM were substantially higher in serum of ovarian cancer patients than in healthy women, suggesting ongoing process of antibody production in ovarian cancer patients. The presence of anti-IL-8 IgG suggests involvement of specific T cells providing help for an isotype switch. It is possible that IL-8 is internalized, processed by B cells or dendritic cells and presented by MHC class II molecules to T helper cells. In patients with ovarian cancer especially at advanced III–IV stage, production of IgM anti-IL-8 Ab reduced resulting in the increase of IgG:IgM ratio.

Our data indicate that simultaneous production of IL-8 and anti-IL-8 autoantibodies results in the formation of complexes.

Table 3
Predictive values for IL-8, anti-IL-8 and CA 125 for early stage ovarian cancer at 98% specificity

Biomarker	αIL-8 Ab	CA 125	IL-8	αIL-8+ IL-8+ CA 125
Sensitivity 95% C.I.	65.5 (63.4, 67.9)	76.8 (75.2,78.5)	62.6 (61.3, 63.2)	87.5 (85.7, 91.1)
%Correctly classified	80.3 (79.8, 80.6)	85.1 (84.6, 85.9)	79.3 (77.9, 80.1)	91.2 (89.6, 92.1)

However, the concentrations of IL-8-antiIL-8 complexes in serum of ovarian cancer patients were slightly lower than of healthy women. The concentrations of these complexes depend not only on the concentrations of IL-8 and anti-IL-8 Ab but also on their absorption by Fc and complement receptors as well as on the rate of elimination of these complexes.

Increased production of IL-8 and anti-IL-8 autoantibodies has been reported in several infectious and inflammatory diseases, such as rheumatoid arthritis, IgA nephropathy, asthma, bacterial meningitis, and acute respiratory distress syndrome (ARDS) [25–33]. IL-8 is involved in the pathogenesis of these diseases [28,34–37]. In asthzma, ARDS, and rheumatoid arthritis, the amount of anti-IL-8 autoantibody complexes with IL-8 correlate with disease severity and outcome [28,30], which suggests a proinflammatory role for these complexes [38]. It has been reported that anti-IL-8 antibody binds IL-8 with a high affinity (approximately 10^{-12} M) [26]. However, the biological significance of autoantibodies to IL-8 is not yet understood. It is possible that anti-IL-8 antibody production represents a physiological mechanism of clearance of excessive IL-8, thus limiting its proinflammatory effects.

In healthy persons and in patients with autoimmune or inflammatory diseases, IL-8 is produced by inflammatory cells. However, in ovarian cancer patients, the main source of serum IL-8 is probably ovarian cancer cells although production by inflammatory cells cannot be excluded. IL-8 is important for stimulation of angiogenesis and tumor cell proliferation and therefore plays an important role in tumor growth and metastasis formation. However, the excessive IL-8 production triggers immune response and formation of anti-IL-8 antibody in ovarian cancer patients that are aimed at elimination of the excess of IL-8 that can disturb normal function of immune system. Furthermore, besides its homeostatic significance, anti-IL-8 antibody could be considered as one of the manifestation of antitumor immune response. Formation of anti-IL-8 antibody might lead to a reduction of tumor produced IL-8 that plays an important role in stimulation of tumor angiogenesis and tumor cell proliferation. It is of interest to test whether anti-IL-8 antibody has a prognostic value in ovarian cancer patients. It is possible to assume that immunostimulation with various immunomodulators might stimulate the antitumor immune response as well as stimulate production of anti-IL-8 antibody leading to a reduction of IL-8 and inhibiting tumor growth.

Therefore, increased production of IL-8 and autoantibodies to IL-8 in ovarian cancer may represent potential biomarkers of the disease. Since IL-8 is not a specific marker it may be necessary to combine it with other cancer biomarkers to increase their predictive value. We demonstrate here that concentrations of anti-IL-8 IgG in patients with ovarian cancer do not correlate with those of IL-8 and CA 125, thus rendering the combination of these markers for a more powerful detection test. Due to a low prevalence of spontaneous ovarian cancer in the general population, a screening strategy must have sensitivity of at least 80% in early stage disease and near perfect specificity of at least 99.6% [39]. Combination of IL-8, anti-IL-8, and CA 125 increased sensitivity to 87.5% at a specificity of 98% of ovarian cancer detection that it is still below the absolute requirement for ovarian cancer detection. Therefore, additional biomarkers are needed in order to further increase the predictive value for early diagnosis of ovarian cancer.

It needs to be noted that the ability of anti-IL-8 antibody to discriminate benign disease is relatively low. Difficulty of discrimination between ovarian cancer and benign pelvic disease is has been previously documented [21]. While this would not preclude use of anti-IL-8 for screening, additional biomarkers need to be identified that provide robust classification of benign pelvic disease from ovarian cancer.

In summary, in the present study, we developed and optimized a new immunofluorescent bead-based assay for detection of anti-IL-8 antibody in blood serum. Our data indicate that serum contains IL-8 cytokine, anti-IL-8 antibody, and IL-8:anti-IL-8 complexes. We further demonstrated that serum of patients with ovarian cancer contains elevated levels of anti-IL-8 antibodies as compared to healthy women. Combining of anti-IL-8, IL-8, and CA 125 resulted in increased diagnostic power of the assay suggesting that circulating antibodies could potentially be valuable diagnostic markers. Further research is underway to analyze the role of multiple circulating antibodies for early detection and prognosis of ovarian cancer utilizing the multiplexing ability of bead-based LabMAP technology.

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research articles Journal of proteome research

High-Sensitivity Blood-Based Detection of Breast Cancer by Multi Photon Detection Diagnostic Proteomics

Andrzej K. Drukier,[†,‡] Nathasha Ossetrova,[†] Elena Schors,[†] Galya Krasik,[†] Ivan Grigoriev,[†] Christina Koenig,[†] Miko Sulkowski,[†] Jerzy Holcman,[†] Larry R. Brown,[‡] John E. Tomaszewski,[§] Mitchell D. Schnall,[∥] Richard Sainsbury,[⊥] Anne E. Lokshin,[#] and Jasminka Godovac-Zimmermann*,[^]

BioTraces Inc., 12455 Sunrise Valley Dr., Herndon, Virginia 20171, OncoTraces Ltd, 3 George Street, London, W1U 3QG, United Kingdom, Department of Pathology and Laboratory Medicine, University of Pennsylvania, 3400 Spruce Street, Philadelphia, Pennsylvania 19104, Department of Radiology, University of Pennsylvania, 3400 Spruce Street, Philadelphia, Pennsylvania, 19104, Department of Surgery, University College London, 67−73 Riding House Street, London W1W 7EJ, United Kingdom, Division of Hematology/Oncology, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213, and Department of Medicine, Rayne Institute, University College London, 5 University Street, London WC1E 6JF, United Kingdom

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We have developed several new methods for blood-based cancer detection by diagnostic proteomics. Ultrasensitive methods of immunoassay using multiphoton-detection (IA/MPD) increase sensitivity by 200- to 1000-fold (1 femtogram/mL). This has allowed the measurement of cancer biomarkers with very low concentrations in blood that could not be measured for full patient cohorts with conventional immunoassays. Sensitivity and specificity in cancer detection have been found to be potentiated by use of immunoassay panels which include tissue-specific cancer biomarkers as well as cytokines and angiogenic factors. The ultrasensitive immunoassays revealed that patient to patient variations in the concentrations of individual biomarkers in blood can extend over many orders of magnitude (up to six) and that the distributions of biomarker concentrations over patient cohorts are non-Gaussian. New methods of data analysis which correlate abundances of multiple, different biomarkers have been developed to deal with such data sets. Sensitivity and specificity of about 95% have been achieved for blood-based detection of breast cancer in pilot studies on 250 patients and 95 controls. Pilot studies indicate that this methodology may also allow differentiation of malignant breast cancer from benign lesions and can provide similar sensitivity and specificity for other epithelial cancers such as prostate cancer, ovarian cancer and melanoma. The methods developed for selection, application, and evaluation of very high sensitivity biomarker panels are expected to have general relevance for diagnostic proteomics.

Keywords: breast cancer • proteomics • immunoassay • blood • biomarkers • multiphoton detection

Introduction

Breast cancer is the most common female cancer worldwide, with an incidence of 1.1 million new cases each year.[1] There are approximately 300−400 thousand deaths a year with the rate varying significantly between countries. Trials have repeatedly, and convincingly, confirmed that breast cancer is a progressive rather than a systematic disease. The stage at which disease treatment is started has a significant impact on clinical outcome and progression of breast cancer can be prevented through early detection and effective early treatment. Early diagnosis, including mammographic screening, is a key factor in the control of breast cancer. In recent years, breast cancer mortality rates have declined as a result of earlier detection and more effective therapy.[2,3] The five-year survival rate for breast cancer is as high as 97% if the cancer is small, of low grade, and has not spread to the lymph nodes.

Currently, diagnosis is by triple testing (clinical examination, imaging with mammography and ultrasonography, and biopsy, either by fine-needle cytology or core biopsy).[4] There remains a need for more effective screening, especially in younger age groups where mammography is less sensitive. Better blood-based testing may aid in early diagnosis, may reduce the need for open biopsy and could provide new modalities for monitor-

* To whom correspondence should be addressed. E-mail: J.Godovac-Zimmermann@ucl.ac.uk.
[†] BioTraces Inc.
[‡] OncoTraces Ltd.
[§] Department of Pathology and Laboratory Medicine, University of Pennsylvania.
[∥] Department of Radiology, University of Pennsylvania.
[⊥] Department of Surgery, University College London.
[#] University of Pittsburgh Cancer Institute.
[^] Rayne Institute, University College London.

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ing of therapy. In addition, post therapy monitoring of patients for recurrence is currently rudimentary and improved methods are required. Many serum biomarkers have been described for breast cancer, but no single biomarker has proven effective. The search for new biomarkers and the simultaneous use of multiple biomarkers is therefore an active area of diagnostics proteomics research.[5,6]

The biology of other epithelial cancers is similar to breast cancer, even if gender, age and the speed of progression are quite different, and there are also unmet needs for better diagnosis of these cancers. For example, ovarian cancer is among the top three causes of cancer-related death.[7] Between 1 and 2% of all women develop epithelial ovarian cancer. The paucity of symptoms and lack of reliable diagnostic modalities mean that most ovarian cancers are discovered at an advanced stage and the overall five year survival rate is about 20%. Prostate cancer is the most common form of cancer among men in the United States,[8] and there is an increasing need for early diagnosis. The standard method for early detection of prostate cancer is screening for prostate specific antigen (PSA). This test, however, has limited specificity and sensitivity and is not specific, so that complementary biomarkers are needed.

Hundreds of proteins have been suggested as putative cancer markers. About 10% are higher abundance proteins and the rest are low abundance proteins. We define as low abundance proteins (LAPs) those proteins for which more than 20% of patients have abundance < 1 pg/mL. There are indications that the low abundance proteins are better biomarkers of cancer, but low abundance proteins could not be reliably measured with prior-art assays. We have solved the problem of assay sensitivity with new immunoassay-multiphoton-detection (IA/MPD) and Super-ELISA assays.[9−11] The dramatically improved assay sensitivity permits use of a multiplicity of low abundance proteins as biomarkers, and permits better than 95% sensitivity (correct detection of cancer) and specificity (correct rejection of healthy) in diagnostic tests for epithelial cancers. Epithelial cancers also cause changes in blood concentrations of angiogenic and inflammatory factors,[12−14] but these proteins (growth factors, cytokines, etc.) typically have very low abundance in blood. In the present paper, we show that immunoassay panels using low abundance proteins that include tissue-specific markers together with angiogenic and inflammatory markers can dramatically improve blood-based diagnosis of breast cancer. However, the extreme, non-Gaussian distribution of serum concentrations of the low abundance proteins over patient cohorts requires nonstandard methods of analysis in order to establish correlations between different biomarkers. Initial data for other epithelial cancers (prostate, ovarian, pancreatic) and other malignancies (melanoma) suggests that similar methods can dramatically improve blood-based diagnostic detection of these cancers. More generally, the present studies reveal new complexities in the selection and application of effective panels of biomarkers, emphasize the need for very high assay sensitivity, demonstrate correlations among different biomarkers and present new methods of data analysis for panels involving correlated biomarkers.

Materials and Methods

Healthy and Breast Cancer Samples. Two sets of serum samples from women with nontreated breast cancer (NT-breast cancer) were studied. One set of samples sets (80 woman samples) were acquired from Zeptometrix, Buffalo, NY. The second (184 woman samples) was obtained from University of Pennsylvania, Philadelphia, PA. Three sets of serum samples from healthy women were studied. The first set of 60 came primarily from young women (20−50 years) who were blood donors at the Hospital Charite, Berlin, Germany. The second set from 30 middle aged American women (age 40−60 years) and the third set from 30 older women (age 60−90 years) came from Zeptometrix, Buffalo, NY. The full set of proteins in SET1 (see text) was measured for only 95 of the healthy samples.

Antibodies and Reagents. Antibodies came from commercial suppliers. For IA/MPD and Super-ELISA, ABs for TNF_α, IL-6 and IL-8 were from CLB, Amsterdam, NL. ABs for PSA were from Alpha Diagnostics, Houston, Texas. ABs for VEGF were from R & D Inc., USA. Antibodies used in the Luminex experiments are described elsewhere.[15,16] ^{125}I labeled streptavidin was obtained from Amersham, UK. Streptavidin-polyHRP was from CLB, Amsterdam, NL. The enzymatic substrate and red-stop were from Neopren, CA, USA.

Read-Out of Immunoassays. Three different methods of signal detection for immunoassays have been used. Immunoassay/multiphoton-detection (IA/MPD) methods used ^{125}I labeled streptavidin.[9−11] MPD-Imager/96 have been constructed by BioTraces Inc., Herndon, VA, USA with a sensitivity of a few zeptomoles per pixel. These imagers are capable of simultaneous MPD measurements in 96 or 384 well formats for microtiter plates.[9] This method gave limits of detection of about 1 femtogram/mL. This proprietary technology of BioTraces Inc. uses ^{125}I to achieve ultrahigh sensitivity in immunoassays. IA/MPD was used to establish very high sensitivity Super-ELISA protocols, which were then used for production measurements of all patient samples. Super-ELISA is similar to IA/MPD except that streptavidin-polyHRP(n), where $n = 20$, 40, or 80, and a colorimetric plate reader are used.[9] Super-ELISA does not use radiolabels, although it is MPD-enabled in the sense that the background-suppression methods developed for IA/MPD have been used to achieve detection limits of 10−50 femtogram/mL depending on the protein. The detection sensitivity of Super-ELISA was adequate to measure serum abundances over virtually all patients for all biomarkers used in the present study (see text).

Luminex Measurements. Using fluorescent, microbead technology from LUMINEX Corporation, the group of Dr. A. Lokshin has developed assays for a wide range of potential biomarkers.. These measurements are described elsewhere.[15,16] The Luminex technique has an estimated limit of sensitivity of about 10 pg/mL.

Data Analysis Procedures. Because the distributions of protein abundances over the cohorts were found to be non-Gaussian, standard statistical packages could not be used to analyze the immunoassay data. A series of programs (300 000 lines of code) which have been written to carry out the data analyses are outlined here and will be described in more detail elsewhere (Drukier et al., in preparation).

Results

Ultrasensitive Immunoassays. Multiphoton-detection (MPD) is an ultrasensitive method for counting of single decay events for isotopes such as ^{125}I that decay by the electron capture mechanism.[9−11] These isotopes can be counted at levels well below background radiation levels and, because single decays are counted, MPD detection is inherently linear over 8−9 orders of magnitude. Immunoassay-multiphoton-detection (IA/MPD) uses ^{125}I labeled streptavidin in sandwich formats to achieve low zeptomole (10^{-21} moles) sensitivity in immunoassays and

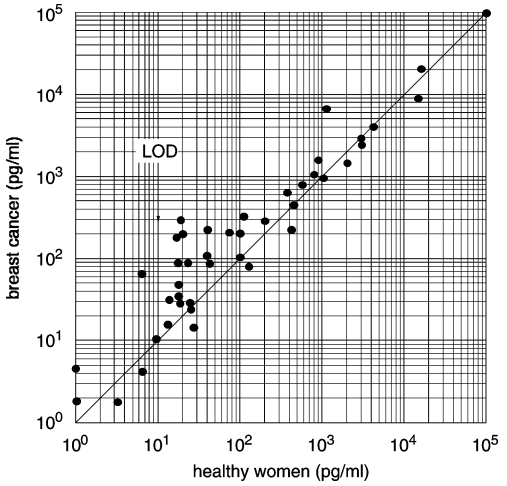

Figure 1. <Average> concentrations of proteins in serum for healthy women (HW) vs breast cancer (BC) patients as measured by Luminex. The proteins include 30 cytokines, chemokines, growth and angiogenic factors (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL- 17, IL-18, TNF_α, TNFRI, TNFRII, DR5, IFN_γ IFN_β, GM-CSF, EGF, VEGF, G-CSF, bFGF, HGF, RANTES, MIP-1_α, MIP-1_β, MCP-1, MIG, and VEGF), 10 putative cancer antigens (CA-125, CA-19−9, CEA, CA-15−3, ErbB2, EGFR, KLK8, Fas, FasL, and Cyfra 21−1), and three matrix metalloproteinases (MMP-2,3, and 7). For non-Gaussian distributions of biomarker concentration with ranges of up to 5−6 logs over a cohort of samples (for both healthy and cancer cohorts, see text), arithmetic averages are highly sensitive to extreme outliers. For a cohort of 100 patients the average can be changed by a factor of 10 by a single outlier. The <average> concentrations shown in the figure are therefore calculated following exclusion of a small number of outliers at greater than 2σ (for both healthy and cancer cohorts). Samples where concentration was below the limit of detection (LOD) were assigned a value of zero in calculating the averages. For some proteins this resulted in the <average> being below the LOD.

requires only about 1 nCi of radioactivity for immunoassays in 96 well microtiter plates.[9−11] Highly effective background suppression is critical to obtaining extreme sensitivity with IA/MPD.

Super-ELISA is similar to IA/MPD except that streptavidin-polyHRP(n), where $n = 20$, 40 or 80, and a colorimetric plate reader are used. Super-ELISA does not use radioactivity, but MPD was essential to optimize the measurement protocols and to reject particular sources of background. IA/MPD typically shows 100- to 1000-fold gains and Super-ELISA shows about 20- to 100-fold gains in sensitivity compared to conventional ELISA assays.[9] This has enabled routine immunoassay of serum proteins that could not be measured by conventional ELISA assays.[9−11] For the first time a wide selection of cytokines could be measured over complete patient cohorts in serum from healthy women or breast cancer patients (see below).

Selection of Biomarker Panels. A wide variety of proteins have been proposed as potential markers of breast cancer. Initial studies were therefore undertaken using the Luminex methodology to identify promising candidates for further studies. Because the sensitivity of the Luminex measurements is about 10 pg/mL, only proteins with relatively high abundance in serum can be analyzed. Figure 1 shows the <average> concentrations of 43 biomarkers in serum for healthy vs breast cancer patients (a small number of extreme outliers have been excluded from the <average>, see the caption to Figure 1). For the majority, the <average> concentrations are largely unchanged, suggesting low predictive power of these biomarkers for breast cancer. For some biomarkers, however, <average> concentrations over a cohort showed significant differences between healthy woman and breast cancer patients. A majority of these promising proteins were clustered near the limits of detection by Luminex (10−50 pg/mL, Figure 1) and could not be measured for all individuals. For example, PSA abundance in serum from females, which is a critical, new marker for breast cancer (see below), is too low for Luminex measurements. Experiments using the more sensitive IA/MPD and Super-ELISA methods, led to the selection of the two panels of biomarkers shown in Table 1 for more detailed investigations.

Typical Protein Distributions from Super-ELISA. Super-ELISA allowed all of the proteins in SET-1 to be measured for virtually all individuals. This coverage of the cohorts was confirmed by IA/MPD for the very few patients where serum concentrations were lower than the detection limits of the Super-ELISA assays (Figure 2A). The need for more sensitive assays is apparent from the limits of detection for conventional ELISA assays. For all biomarkers except VEGF, conventional ELISA assays can only measure the limited portions of the patient cohorts that show high abundances in serum (Figure 2A). Furthermore, even with the high sensitivity and accurate quantitation of Super-ELISA, no single biomarker showed a complete separation between healthy and breast cancer patients (Figure 2A).

Statistical analyses of such data often assume that biomarker abundances show Gaussian distributions around an average, but the data for the full cohorts clearly indicated that Gaussian distributions are not valid for the five markers measured by Super-ELISA (note the log scale in Figure 2A). Furthermore, there are appreciable changes in the averages as a function of age for both healthy and breast cancer patients (Figure 2B). The age-related changes might be a reflection of the menopausal status of the women, which was not available for the samples used.

Two-Dimensional Clustering. The above results indicated that the abundances of the individual proteins cannot be a reliable cancer diagnostic. If serum concentrations of these proteins are to be used, then correlations between multiple markers will be needed and due consideration of patient age

Table 1. Panels of Biomarkers Tested for Breast Cancer Diagnosis

			protein markers assayed		
panel	detection	cohort[a]	disease markers	inflammatory markers	angiogenic markers
SET 1	super-ELISA	160 BC, 95 HW	PSA	TNFα, IL-6	IL-8, VEGF
SET 2	luminex	53 BC, 60 HW	CA-125, CA-19.9, CK19 EGFR, MMP7	TNFα, IL-6, IL-15	IL-8, VEGF, EGF

[a] Number of breast cancer patients (BC) and number of healthy patients (HW).

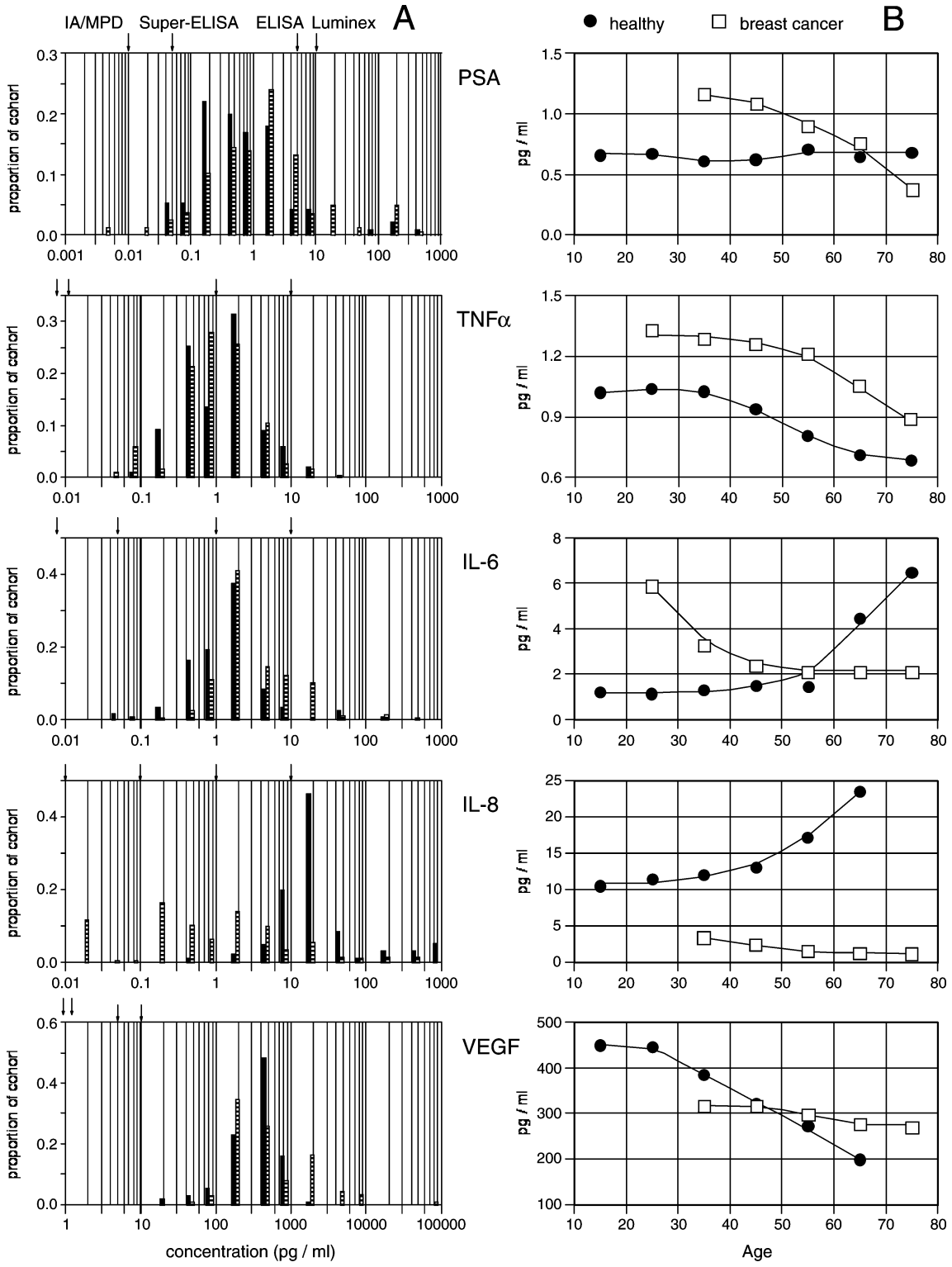

Figure 2. (A) Distributions of the serum concentrations of PSA, IL-6, IL-8, TNF_α and VEGF for healthy women (solid bars) and breast cancer patients (hatched bars). The arrows at the top of each plot indicate the sensitivity of Luminex, conventional ELISA assays, Super-ELISA and IA/MPD. (B) Age dependence of the <average> concentrations in serum of PSA, IL-6, IL-8, TNF_α, and VEGF for healthy women (HW) and breast cancer patients (BC).

will be required. Measurements of multiple protein concentrations inherently involve multidimensional data (5 and 11 markers imply 5 and 11 data dimensions for biomarker SETs 1 and 2 respectively). In principle such data sets can be analyzed in high-dimensional spaces to search for generalized ellipsoids characteristic of healthy and breast cancer patients, but such analyses are difficult to visualize or to understand intuitively. We have therefore considered the protein concentrations in pairs, where correlations between different proteins can be readily observed. For N-biomarkers there are $N(N-1)/2$

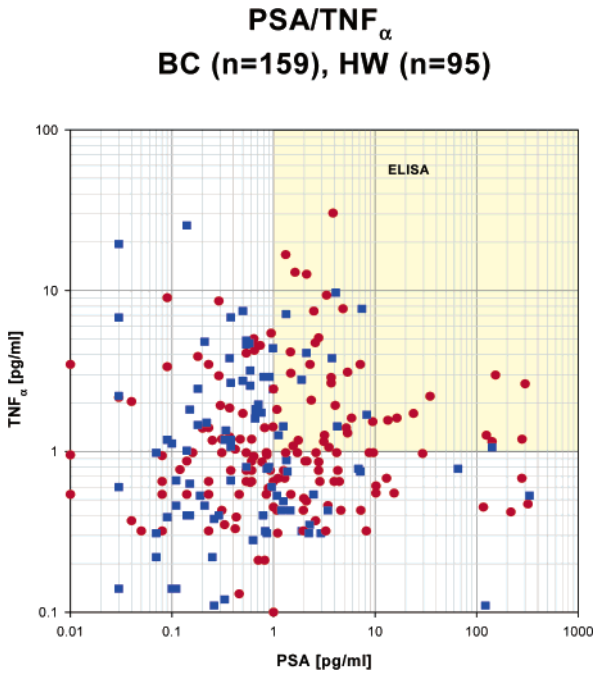

Figure 3. Correlation between the concentrations of PSA and TNF$_\alpha$ in serum of healthy women (blue squares) and breast cancer patients (red circles). The yellow shaded region indicates data points that are measurable with conventional ELISA assays.

different pairwise combinations of two biomarkers, which meant that 10 and 55 two-dimensional planes were examined for biomarker SETs 1 and 2.

Figure 3 shows the distribution of the pairwise concentrations of PSA and TNFα. Several fundamental characteristics of the full data sets are already apparent. First, conventional ELISA or Luminex-based assays can only cover a small part of the two-dimensional plane, i.e. the very high sensitivity of Super-ELISA becomes essential for obtaining high cohort coverage when considering correlations between different biomarkers. Second, there are very wide variations in the protein concentrations in serum of different patients for both PSA (5 orders of magnitude) and TNFα (3 orders of magnitude). Finally, in two dimensions the patient data is scattered over the plane and there is no complete separation between healthy and breast cancer patients. Patients with breast cancer show pairs of (PSA TNFα) concentrations where both proteins are either high or low compared to the average concentrations.

Composite Scores from Two-dimensional Correlations. For the five proteins assayed by Super- ELISA, there are a total of 10 possible pairwise, two-dimensional correlation planes. Each biomarker in such a correlation plane has been age corrected and the concentration scales renormalized to better handle data points spread over several orders of magnitude. Each plane is clustered into regions in which either healthy woman or breast cancer patients are the predominant, but not exclusive, population. In areas of the planes with significant overlap between healthy woman and breast cancer patients, an "uncertain" region has also been assigned. Two such planes are shown in Figure 4A. Each of these two-dimensional planes can be regarded as an independent assay for detection of breast cancer. However, they are not "cutoff" assays − both very high and very low abundances of individual markers can be an indication of breast cancer.

Following clustering, all of the 2D planes were used to calculate a composite score. For each 2D plane, the data from a single patient was assigned a value of +1 (predominantly breast cancer region), 0 (uncertain region) or −1 (predominantly healthy region). For an individual patient, the composite score is the sum over the 10 planes. If all 10 biomarker pairs fall in regions in the 2D correlation planes assigned to predominantly cancer, then the patient would be assigned a composite score of +10. Following classification of all patients, the composite scores were normalized to the range − 100% to +100% (essentially division by the number of biomarkers in the panel), which provides a common scale independent of the number of biomarkers. Ideal separability would imply that all healthy women would have a strongly negative composite score and all women with breast cancer would have a strongly positive composite score. As shown in Figure 5, most beast cancer samples have a composite score (CS) > 80% (152 out of 159 samples), whereas most samples from healthy women have a composite score < −40% (92 out of 95 samples). There is only a small overlap, involving a few percent of patients, in the range between −20% and +20%.

We have also explored alternative methods for assigning composite scores to patients. In particular, it is possible to use methods such as wavelet processing so that continuous values (not just ± 1) are assigned to data points in the two-dimensional planes (Figure 4B). This has advantages for recognition of interspersed regions of healthy and breast cancer in the planes (see the plane for PSA/VEGF in Figure 4B) and is discussed in more detail elsewhere.

Overall, the panel of markers permits detection of breast cancer in almost all women (> 98%) for which cancer has been previously detected by biopsy, mammography or MRI (BC cohort). Correlations of our blood-based assay with biopsy, MRI and mammography are remarkably good, and will be discussed elsewhere. Importantly, 95% of healthy women were properly identified. In early disease detection, the FDA requires the provision of so-called ROC curves. Typically ROC curves at 80% are acceptable and curves at 85−90% are regarded as excellent. The present results correspond to ROC values of about 95%, which is about 10% better than any prior-art breast cancer detection method, including mammography. In short, ultrasensitive immunoassays that provide data for all patients in a cohort together with the use of correlations between appropriate pairs of biomarkers substantially improves classification of breast cancer.

How Many Biomarkers are Needed? Not all biomarker pairs are equally powerful in separating healthy and breast cancer patients (Figures 3,4). To take this into account, the influence of different planes on the specificity and sensitivity of the classification of the patients has been examined. For each plane, the predictive power is defined as the average of the probability of correct classification of healthy (specificity) or of breast cancer (sensitivity) patients. Table 2 shows the predictive powers of the different planes.

Essentially, there are three classes of such 2D correlation functions, with predictive power (PP) of PP < 70%, 70% < PP < 80% and PP > 80%. For example, in the case of breast cancer diagnostics, PSA/TNFα has a medium predictive power of less than 70%. The regions of this plane which are populated by the BC cohort are closely interspersed with regions containing members of the HW cohort (Figure 3). IL-6/IL-8 represents a 2D correlation plane with about 80% predictive power (see Figure 4A). In this case, there are several regions populated by

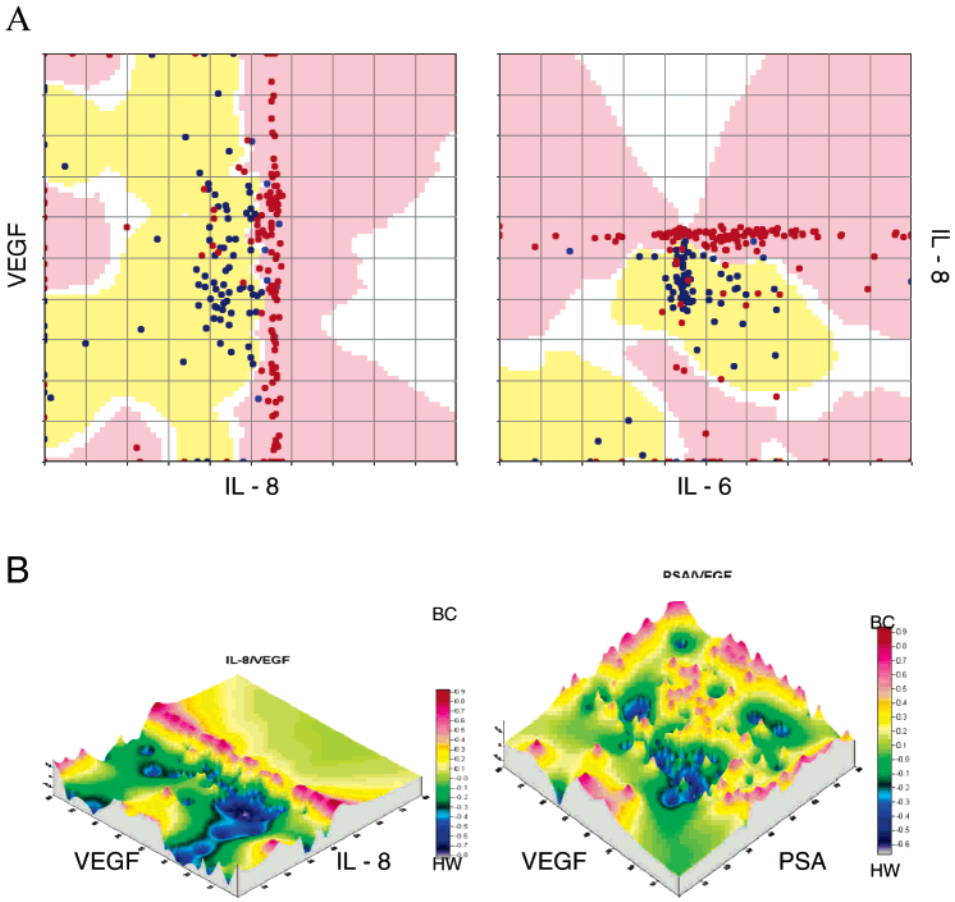

Figure 4. A. Age-adjusted and renormalized two-dimensional correlation planes for the protein pairs IL-6/IL-8 and IL-8/VEGF as measured by Super-ELISA. Healthy/cancer patients correspond to blue/red circles, respectively. The assigned regions are healthy (yellow), breast cancer (red) and uncertain (white). B. Wavelet processing of the 2D correlation data for the pairs PSA/VEGF (~85% predictive power) and (IL-8/VEGF (~95% predictive power). The behavior of each pair is typical for pairs with similar predictive power, e.g., at 85% predictive power, isolated regions for healthy patients are interspersed with cancer regions.

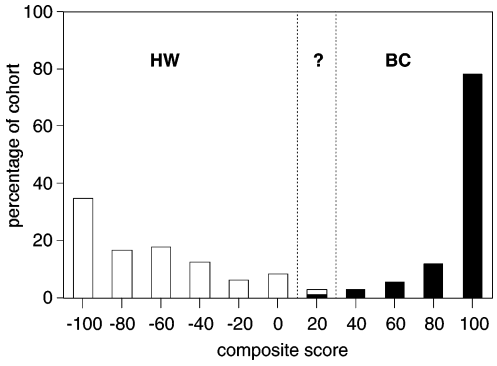

Figure 5. Percentage of healthy (HW) and breast cancer (BC) patients vs the composite score.

the HW cohort, but these regions are clustered and relatively well separated from the regions where the BC cohort dominates. Finally, the IL-8/VEGF plane has a predictive power of about 95%. This landscape shows overlapping regions for the HW cohort and two well-defined ridges for the BC cases (Figure 4).

The predictive powers and their associated landscapes suggest that some of the markers may be less important, e.g., TNFα does not appear in the very high classification for either specificity or sensitivity. For cost and efficiency of analysis it is desirable to use the smallest number of biomarkers that is consistent with adequate levels of classification and we have therefore examined how the elimination of individual markers influences the final classifications (Table 2). PSA has a strong role in specifying the cancer as breast cancer and therefore cannot be removed (see below). Removing IL-6, IL-8 or VEGF leads to substantial decreases in specificity, i.e., the number of uncertain and/or false classifications increases considerably. The data suggests that the biomarker with the lowest predictive power is TNFα. When all measurements of TNFα are removed, there is a only few percent reduction in the specificity of the biomarker set. While this suggests that TNFα might be removed from the panel, we have observed that planes such as IL-6/TNFá help to detect differences in immune responses for different kinds of cancer (see below).

Table 2. Predictive Power[a] of Different Two-dimensional Correlation Planes

classification	2D correlations
low (<70%)	PSA/TNFα; PSA/IL-6; TNFα/IL-6
high (70−80%)	PSA/IL-8; TNFα/IL-8; TNFα/VEGF
very high (> 80%)	PSA/VEGF; IL-6/IL-8; IL-6/VEGF; IL-8/VEGF

[a] Predictive power is defined by: $PP = 100(1 - (N_f + N_p)/N)$, where N is the total number of data points, and N_f and N_p are the numbers of false negatives and false positives in the assignment of the data points to cancer or healthy.

Tests of Robustness. Several tests of the robustness of the classification method have been made:[1] dependence on the precision of the data measured for individual patients,[2] dependence on the assay sensitivity, i.e., the proportion of the cohort for which data can be measured,[3] dependence on training set, i.e., the inclusion or exclusion of data for individual patients, and[4] dependence on the exact algorithms used for clustering of the data to regions of healthy, uncertain or breast cancer. First, the different assay methods have somewhat different precision. For Super-ELISA the measurement variance is 20% for < 0.1 pg/mL, 15% for 0.1−1 pg/mL and 5−10% for > 1 pg/mL. For ELISA it is 20% for 1−10 pg/mL and 10−15% for > 10 pg/mL. For Luminex it is 20−50% for 10−50 pg/mL and 10−30% for > 50 pg/mL. By propagating random errors of these magnitudes through the data sets, we found that the precision of the data has only a very moderate influence on the classifications. This is not surprising since the two-dimensional correlation planes cover many orders of magnitude and are very sparsely populated. Small changes in measured values do not move the points significantly in the 2D planes. Second, the specific clustering and renormalization algorithms also have only a limited influence on the classifications—here again the availability of multiple, sparsely populated 2D planes seems to be decisive. Third, the size of the data sets used in the present study was shown to be adequate in two ways. Random choice of different patients showed that for cohorts with about 40 BC and 40 HW samples, the resulting classifications were stable and gave specificity and selectivity > 95%. Furthermore, when the available data was randomly separated into a training cohort of 40 BC, HW patients and the resulting 2D planes were used to classify the remaining samples (validation cohort), specificity and selectivity was also > 95%. These analyses will be described in greater detail elsewhere (Drukier et al., in preparation).

However, the assay sensitivity (LOD) can strongly influence separability of the data into healthy and disease cohorts since insufficient sensitivity reduces the number of two-dimensional planes that can be used for classification of individual patients. The very different sensitivities of the four measurement techniques (LODs of: IA/MPD 1−5 fg/mL; Super-ELISA 10−50 fg/mL; ELISA 1−5 pg/mL; and Luminex, 10−50 pg/mL) results in marked differences in the proportion of patient samples that give correlated pairs of measurement values. Whereas both IA/MPD and SUPER-ELISA allow detection of over 95% of the 2D correlations in the patient cohorts, this is reduced to 50% for ELISA and 20% for Luminex (where only 40% of the IL-8, VEGF pairs remained). Concomitantly, the sensitivity the sensitivity and specificity were both reduced by about 5−10% (ELISA) or by 15% (Luminex). Typically this results in overlap of the composite scores for the different cohorts, producing data that is not interpretable (Figure 6).

Finally, we have used the Luminex data to assess whether increased numbers of biomarkers can compensate for reduced patient coverage due to lack of adequate measurement sensitivity. We focused on postmenopausal women with associated mammography tests (BC: $n = 53$; HW: $n = 60$). Only some of the biomarkers appeared likely to have useful predictive power (Figure 1). A previous analysis of the Luminex data by biostatisticians at the University of Pittsburgh achieved cancer vs healthy identification with sensitivity of about 75% and specificity of about 70%, but more than 16 biomarkers were needed (A. Lokshin, private communication). We selected a much smaller set of 11 biomarkers for detailed analysis with the more

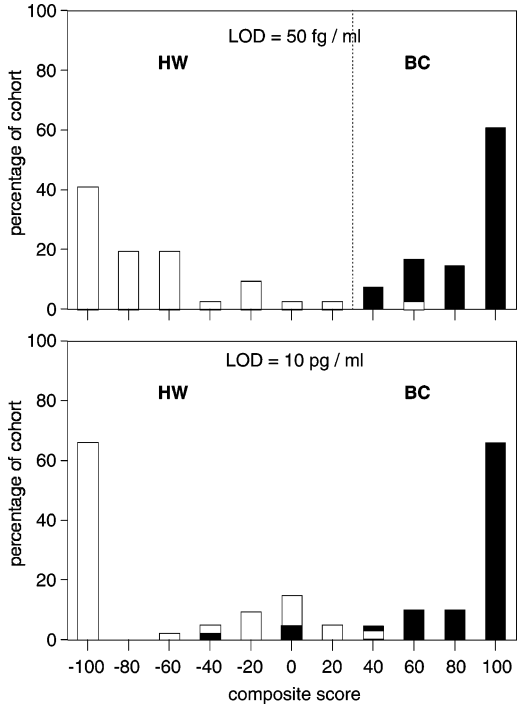

Figure 6. The effect of assay sensitivity on the correlation analysis, in particular the effect of missing data from unmeasurable serum levels. Top: Using Super-Elisa all data points can be measured. All 2-D planes can be clustered and have good quality that results in good separation between healthy and cancer cohorts. Bottom: Using a less sensitive assay, some data points are missing. The 2-D planes have lower quality or cannot be clustered. This leads to lack of separation of the cohorts.

sophisticated correlation-based algorithms. The panels of biomarkers used in the Luminex analyses include tissue markers, immune response markers and angiogenic factors (Table 1). Indeed, the Luminex markers include the cytokines and angiogenesis factors (TNFα, IL-6, IL- 8, and VEGF) used in the Super-ELISA analyses. Table 3 shows the percentages of correct, uncertain and false classifications of patients using these data sets with the 2D correlation methods. The healthy and breast cancer patients could be largely separated and sensitivity and specificity better than 90% could be obtained (Table 3). Similar analyses have also been done with other types of cancer and are considered further below (see discussion). However, while these results look promising, the data sets are not large enough to be statistically certain and may not be stable with regard to random choice of patient subsets. For example, the 2D correlation of CA19−9/TNFα was found to have predictive power of 95%, but could only be measured for less than 10% of patients with Luminex (Figure 2).

Extensions of the Two-Dimensional Correlation Analysis. The ultimate goal of a measurement on an individual patient is a diagnosis, which may require decisions both about what type of cancer is present and whether the cancer is benign or malignant. Preliminary studies indicate that 2D correlation analyses may also be useful in these contexts. Figure 7 shows initial results on using an 11 biomarker analysis to distinguish benign/malignant breast cancer. Noteworthy is that no false assignments are made and that the number of patients classified as uncertain is small. Figure 8 shows very promising initial results on using an 11 biomarker analysis to distinguish

Table 3. Summary of Cancer Classification Using Two-Dimensional Correlation of Biomarkers

method/patient cohort	no. of biomarkers	correct identification	uncertain identification	false identification
		Using Super-ELISA		
breast cancer ($n = 264$)	5	252/264 (95.4%)	5/264 (1.9%)	7/264 (2.7%)
healthy women ($n = 95$)		89/95 (93.6%)	4/95 (4.2%)	2/95 (2.2%)
prostate cancer ($n = 32$)	6	32/32 (100%)	0/32 (0.0%)	0/32 (0.0%)
BPH men ($n = 60$)		58/60 (96.7%)	0/60 (0.0%)	2/60 (3.3%)
prostate cancer ($n = 32$)	6	30/32 (93.8%)	0/32 (0.0%)	2/32 (6.2%)
prostatitis men ($n = 19$)		19/19 (100%)	0/19 (0.0%)	0/19 (0.0%)
		Using Luminex		
breast cancer ($n = 63$)	11	63/63 (100%)	0/63 (0.0%)	0/63 (0.0%)
healthy women ($n = 56$)		56/56 (100%)	0/56 (0.0%)	0/56 (0.0%)
ovarian cancer ($n = 51$)	11	48/51 (94.1%)	2/51 (3.9%)	0/56 (0.0%)
healthy women ($n = 56$)		54/56 (96.4%)	2/56 (3.6%)	1/51 (0.0%)
pancreatic cancer ($n = 72$)	12	71/72 (98.6%)	0/72 (0.0%)	1/72 (1.4%)
healthy individuals ($n = 53$)		53/53 (100%)	0/53 (0.0%)	0/53 (0.0%)
melanoma ($n = 172$)	9	164/172 (95.3%)	7/172 (4.1%)	1/172 (0.6%)
healthy individuals ($n = 100$)		96/100 (96.0%)	3/100 (3.0%)	1/100 (1.0%)
All Cancers ($n = 591$)		567/591 (95.9%)	14/591 (2.4%)	10/591 (1.7%)
All Healthy ($n = 383$)		369/383 (96.3%)	9/383 (2.3%)	5/383 (1.4%)

breast and ovarian cancers. These data are considered further in the discussion.

Discussion

The starting concept for the present experiments was the notion that blood-based detection of cancer would be potentiated by the measurement of markers of systemic response in addition to tissue-specific disease markers. The hypothesis was that tissue/disease biomarkers permit high sensitivity and that additional inflammatory and angiogenic biomarkers will improve the specificity. There is a substantial body of literature that motivates this idea, but it could only be tested reliably with the high sensitivity assays used here. For example, the immune response to cancer is an important defense against epithelial cancers, including breast cancer, and the present results support previous indications that measurement of the immune response helps in the detection of breast cancer. TNFα and IL-6 tend to be elevated in serum, as well as ascites, of women with breast cancer.[17] It has previously been reported that serum levels of IL-8 are significantly elevated for epithelial cancers compared to normal controls[18] and that levels of IL- 6 are higher in epithelial cancers than in benign tumors.[18] In addition to inflammation, cytokines such as IL-1, IL-6, and TNFα are also involved in some oncogenic processes.[19] Considerable work on cell lines suggests the importance of pro-inflammatory cytokines in ovarian cancer,[20] but in-vivo studies have been much more difficult due to the very low levels of cytokines in blood. Thus, altered serum levels of cytokines are certainly associated with breast cancer, as they are with other cancers.

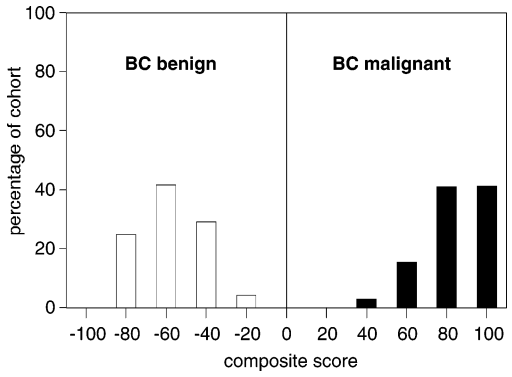

Figure 7. Percentage of patients with benign (open bars) or malignant (solid bars) breast cancer versus the composite score.

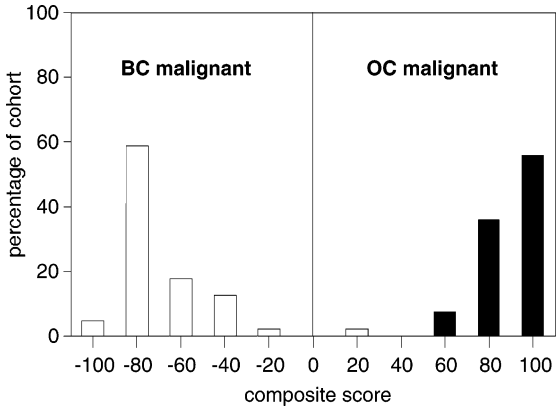

Figure 8. Percentage of patients with malignant breast cancer (open bars) or with malignant ovarian cancer (solid bars) versus the composite score.

Similarly, angiogenesis, i.e. the development of new blood vessels, is an essential component of solid tumor growth and metastasis.[21] Angiogenic factors are expressed by many tumors and activate host epithelium.[22] The formation of the vascular stroma is crucial in the pathophysiology of malignancy and the onset of angiogenesis can mark a phase of rapid proliferation, local invasion and ultimately metastasis.[23] Vascularisation also allows greater accessibility for stray tumor cells.[24] The importance of angiogenesis factors in tumor growth thus suggests that they are potentially useful biomarkers for cancer. Vascular endothelial growth factor (VEGF) is a highly potent angiogenesis factor that plays a coordinated role in endothelial cell proliferation. Other important angiogenesis factors include IL-8, fibroblast growth factor (FGF) and the platelet derived endothelial growth factor (PD-EGF). Because these factors act locally at the sites of vessel formation, there is no particular barrier between them and blood. Local changes in their abundance are usually reflected in global changes in blood abundances, but these may be up or down. For example, the strong chemoattraction of a tumor can lead to local enhancement of IL-8 concentration at the tumor site, with a reduced concentration in blood (A. K. Drukier, unpublished results).

Thus, angiogenic factors provide excellent biomarkers for tumor formation and this has been observed in the present studies—for breast cancer, the serum abundances of IL-8 and VEGF tend to decrease and increase respectively.[25]

Prostate specific antigen (PSA) was initially believed to be specific to the prostate and has long been used as a marker of prostate cancer.[26,27] In men, PSA is produced mainly, but not exclusively, by the prostate, and PSA blood levels are much higher in men than in women. Most PSA in the blood is bound to proteins that inhibit the proteolytic activity of PSA and high levels of the free enzyme are more suggestive of cancer than high total levels.[26] PSA has also been found in various female tissues and body fluids. Female breast, both normal and cancerous, produces PSA,[28] and this production is regulated by estrogen and progesterone. Preliminary data suggest that women with breast cancer may have a better prognosis if the level of PSA in tumors is high. A study which examined the prognostic value of PSA in a large cohort of US patients, using an assay with a detection limit of a few pg/mL, measured the level of PSA in tumor cytosolic extract of 953 women with preliminary breast cancer diagnosis.[14] Detectable PSA, i.e., PSA levels > 5 pg/mL, were found to be significantly associated with smaller tumors, tumors with a small *s*-phase fraction, diploid tumors, younger age, and tumors with lower cellularity. Reduced risk for relapse and death remained statistically significant after accounting for other clinical and pathological variables. Because of the need for breast extracts and the limited sensitivity of the available immunoassays for PSA, these studies could not be extended to use of PSA in early diagnostics of breast cancer. We have developed assays for PSA with at least 100-fold improved sensitivity and were able to show that PSA abundances in blood provide a new tissue/disease biomarker for early breast cancer diagnosis.

Concurrent use of tissue-specific disease markers as well as markers of systemic response is strongly supported by the present results. However, the experiments presented new technical challenges, especially the requirement for more sensitive immunoassays to measure low abundance cytokines and angiogenic factors. Super-ELISA and IA/MPD assays have adequate sensitivity to monitor complete patient cohorts for PSA, IL-6, IL- 8, TNFα, and VEGF, whereas the complete cohorts could be monitored only for VEGF with prior-art ELISA assays (Figure 2). The new data on PSA, IL-6, IL-8, and TNFα indicates that prior-art ELISA assays were monitoring only fractions of the complete range of abundances of these proteins in both healthy woman and cancer patients. In the case of PSA and IL-8, the sensitivity of prior ELISA assays failed to reach even the average abundance seen for the patient cohorts (Figure 2). Consequently, pairs of biomarkers such as PSA/IL-8 were only very poorly covered by the sensitivity achievable with prior-art ELISA and pairs of biomarkers such as IL-8/IL-6 that have been found to have very high predictive power for breast cancer (Table 2) were mostly inaccessible without the sensitivity of Super-ELISA.

At present, the Luminex data are mainly available for a small cohort of post-menopausal women. Much increased cohort sizes will be needed to clarify the preliminary indication that measurement of large numbers of biomarkers can at least partially offset incomplete cohort coverage. Alternatively, Luminex may be particularly valuable in a "directed-discovery" mode in which sizable groups of putative biomarkers are assessed for diagnostic potential. The greater patient coverage, but lower throughput of Super-ELISA will be useful in validating biomarkers and essential for practical diagnosis of individual patients. The performance of the present blood-based assays for breast cancer strongly supports the use of supersensitive assays for cytokines and angiogenic factors to reduce the number of false positives in cancer diagnosis.

The importance of using correlated biomarkers is also supported by our other initial results. For example, the correlation methods show promising preliminary results for distinguishing benign/malignant breast cancers (Figure 7). This task normally requires biopsies that are costly, manpower-intensive and prone to stress/pain, i.e., a blood-based means of diagnosis would have major advantages. Similarly, the correlation methods show attractive initial results for differentiation between different types of cancers (Figure 8). Indeed, initial studies with smaller cohorts indicate that the two-dimensional correlation methods can provide high sensitivity and specificity for diagnosis of other cancers (Table 3). This suggests that the correlation methodology shows high promise for pan-cancer diagnostics. We are now exploring how many and which combinations of biomarkers would be needed for a biomarker panel capable of concurrently handling multiple types of epithelial cancers. Specific tissue/disease biomarkers are clearly needed, e.g., we have found that the combination of MIP-1a, MIP-1b, and MPA distinguishes melanoma from other cancers. On the other hand, initial indications are that there are changes in the serum abundances of cytokines and angiogenic factors that are distinctive for different cancers. For example, correlation planes for pairs of biomarkers such as IL-6/IL-8 and IL-8/VEGF are significantly different depending on the type of cancer and this could reduce the number of biomarkers that are needed for pan-cancer diagnostics. Alternatively, we have already optimized Super-ELISA assays for a wide range of other potential biomarkers that may be useful in parallel assays for different types of cancers.[9]

Finally, we suggest that the results obtained in the present work have important implications for diagnostic proteomics in general, especially in the selection, application and evaluation of biomarker panels. There is now abundant evidence that single, golden-bullet biomarkers of diseases are very unlikely and that multiple biomarkers are essential for diagnostic proteomics. The present studies show conclusively that in breast cancer patients the same biomarker may have either high or low serum concentrations relative to the averages over patient cohorts and that predictive power is considerably improved when the abundances are used in correlation with other biomarkers. This strongly suggests that it is not the presence of a given protein, but rather it's participation in particular networks of proteins and functions that has a high predictive power for a particular disease. It is not surprising that there are correlations between different biomarkers in the physiology of breast cancer and similar behavior is highly likely for other diseases. The present 2D correlation methods provide an effective way of exploiting such correlations for diagnostic purposes and avoid the clearly inadequate assumption of averages and Gaussian statistics to describe and interpret biomarker concentrations for patient cohorts. Selection of efficient biomarker panels is likely to require judicious combination of high-throughput screening methods, very high sensitivity assay methods and, perhaps increasingly, targeted exploitation of knowledge about physiology. Present experience suggests a number of features that should be considered in designing effective panels of biomarkers.[1] Low abundance

proteins can be excellent biomarkers and may be more informative than high abundance proteins.[2] Combination of tissue/disease specific markers with markers of systemic response may be particularly effective.[3] Validation of biomarkers is greatly facilitated if the biomarker is measurable over full patient cohorts.[4] The panel should include combinations of biomarkers with clear correlations between different biomarkers.[5] Biomarker combinations are especially sensitive to cohort coverage and efficient diagnosis of individual patients requires very high cohort coverage for the individual biomarkers. In addition, such panels need to be shown to be robust and disease specific. For this purpose new methods of data analysis will be needed. We believe that the methods and results obtained in the present work represent an important step toward achieving accurate, correlated measurements of the abundances of multiple, informative biomarkers that will be essential to moving beyond "diagnostic proteomics" and toward "functional proteomics" that is closely related to physiology and pathology.

Abbreviations. MPD, multiphoton-detection; IA/MPD, immunoassay with multiphoton- detection read-out; Ab, antibody; polyHRP: poly horseradish peroxidase; TNFα, tumor necrosis factor α; TNFRI, II, TNF Receptors 1 and 2; IFNγ, interferon gamma; EGF, epithelial growth factor; VEGF, vascular endothelial growth factor; EGFR, epithelial growth factor receptor; CK19, cytokeratine 19 kDa; MMP-2, -3, -7: matrix metalloproteinase 2.3.7; PSA, prostate specific antigen: FDA, Federal Drug Administration; ROC, Receiver Operating Characteristics.

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